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MODE OF ACTION OF MEMBRANE PERTURBING AGENTS: SNAKE VENOM CARDIOTOXINS AND PHOSPHOLIPASES A

ANNUAL REPORT

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Cardiotoxins (CTXs) are potent membrane perturbing agents that interact in mutual synergism with phospholipase A2 (PLA2). The CTXs selectively lyse a subpopulation of older red blood cells (RBCs), as determined by similarities between hemoglobin and ^{3}H-deoxyglucose-6-p release. This lysis is enhanced by low concentrations of Ca^{2+}, through activation of PLA2 activity, and inhibited by high concentrations of Ca^{2+}. Hemolysis is antagonized completely by Mn^{2+} and competitively by low concentrations of sphingosine and polymyxin B. Other protein kinase C and calmodulin inhibitors are ineffective antagonists of CTX action. A PLA2 with CTX-like properties irreversibly alters the response of Ca^{2+}-regulated voltage-dependent K^+ channels by locking on a Ca^{2+}-dependent process, despite only a slight transient increase in intracellular Ca^{2+} levels. Melittin, a model for CTX action, can remove barriers blocking the membrane penetrating capability of PLA2. Melittin and CTX fractions contaminated with even trace PLA2 activity have dramatic effects on fatty acid release that could wrongfully be interpreted as activation of an endogenous (tissue) PLA2. Effects of both of these agents on tissue lipase activity will be examined in year three.

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Problem Under Study:

Contract No. DAMD17-87-C-7155 addresses the mode of action of snake venom cardiotoxins (CTXs) and the interaction of these toxins with a second snake venom component, phospholipase A_2 (PLA_2). Studies on presynaptically-acting snake venom PLA_2 neurotoxins examine whether similarities exist in mechanisms between these toxins and the CTXs, or if the CTXs act synergistically with the PLA_2 neurotoxins. CTXs are potent membrane perturbing agents and PLA_2s hydrolyze diacylphosphoglycerides at the two position, generating two biologically active metabolites - free fatty acids and lysophospholipids. The CTXs and PLA_2s act in synergy to induce hemolysis of red blood cells and skeletal muscle contractures. The CTXs and PLA_2s are of special interest due to the mutual potentiation observed; that is, the hemolytic activity of CTX is greatly increased by PLA_2 and the hydrolytic activity of PLA_2 is greatly increased by CTX. Combinations of these agents with other toxins, such as the presynaptically-acting snake venom neurotoxins, which all possess PLA_2 activity, would result in novel potent biological warfare approaches. Additionally, based on our recent studies, there are similarities between the modes of action of CTX and presynaptically- acting snake venom toxins. Therefore, a greater understanding of the modes of action of venom CTXs and PLA_2s and their interactions has important military significance.

The specific problems addressed in this contract are:

1. What are the effects of CTX alone and CTX-PLA_2 combinations on contracture induction of human and rat skeletal muscle and on Ca^{2+} transients in human and equine lymphocytes?
2. What is the dependence of CTX action on fatty acid distribution and the free fatty acid content of muscle and red blood cells?
3. What are the hemolytic effects of CTX alone and CTX-PLA_2 combinations in red blood cells from different species?
4. What are the effects of CTX on endogenous lipolytic enzymes in skeletal muscle and red blood cells?
5. What is the role of toxin internalization in the action of CTX?
6. What are the similarities and differences between CTXs and the presynaptically-acting snake venom PLA_2s?

Background:

Early studies in the isolation of protein components from snake venoms identified a number of toxic low molecular weight polypeptides (ca. 6000 MW). These toxins were identified in a number of laboratories and, because they affected a large number of systems, some confusion arose as to the true site of action of the toxins. The polypeptides were named according to their observed toxic actions and included: cardiotoxins, which arrested the heart in systole (Sarkar, 1951; Lee et al., 1958); Cobramine A and B (Larsen and Wolff, 1968); cytotoxins (Braganca et al., 1967) and; direct-lytic factors (Condrea et al., 1964). It later became apparent that these seemingly different toxins indeed shared similar mechanisms when compared on the same assay systems. The multitude of different effects possessed by each of these toxins were suggested to be best described by the more general term membrane-active polypeptides (Condrea, 1974). However, the toxins have since been

grouped together under the original name, cardiotoxin (CTX). CTXs are now regarded as low molecular weight basic polypeptides that, among other effects, depolarize and induce contractures in muscle (Chang, 1979) and hemolyze red blood cells (Condrea, 1974; 1979). There appears to be considerable amino acid sequence homology among the CTXs (Condrea, 1974). Despite considerable research in this area, the specific mechanism(s) of action of the CTXs is(are) unknown (Chang, 1979; Harvey; 1985).

Phospholipase A_2 (PLA_2; EC3.1.1.4; ca. 12,000 MW) is a major component of bee and snake venom. PLA2 is a Ca^{2+}-dependent enzyme (Shipolini et al., 1971; Tsai et al., 1985) that catalyzes the hydrolysis of phospholipids at the #2 position releasing lysophospholipids and, primarily, unsaturated free fatty acids (Hanahan et al., 1960). The bee and cobra (*Naja naja*) venom PLA_2 enzymes readily hydrolyze biological phospholipid substrates, but are unable to penetrate membrane bilayers (Zwaal et al., 1975; Sundler et al., 1978; Fletcher et al., 1987). The inability to hydrolyze the inner phospholipids of the bilayer does not relate to interactions of these primarily negatively-charged phospholipids with spectrin (Raval and Allan, 1984), and can even be observed in pure phospholipid vesicles (Sundler et al., 1978; Wilschut et al., 1979). Hemolysis of fresh human red blood cells is not induced to any significant extent by bee venom PLA_2, even though almost all the phospholipids in the outer leaflet of the membrane bilayer have been hydrolyzed (Zwaal et al., 1975; Fletcher et al., 1987).

Louw and Visser (1978) reported that some CTX fractions were contaminated with trace amounts of venom PLA_2, which greatly potentiated the hemolytic activity of the CTX protein. Trace contamination of crude CTX preparations with PLA_2 activity has confounded the interpretation of some of the toxic actions of CTXs, especially when tested on hemolysis of erythrocytes (Harvey, 1985; Louw and Visser, 1978; Harvey et al., 1983). Highly purified CTXs are considered virtually devoid of PLA_2 contamination and have considerably reduced hemolytic potency compared to PLA_2 contaminated preparations. However, the potency of CTX on other preparations, such as skeletal muscle, is unaffected by PLA_2 contamination. Therefore, PLA_2 activity has been considered to be essential for the hemolytic action of CTXs on erythrocytes, suggesting the mechanism of action of CTX on the red blood cell does not reflect the same mechanism in skeletal muscle (Harvey, 1985). However, more recent studies have demonstrated that higher concentrations of PLA_2 do act in synergy with CTX in skeletal muscle and that the <u>apparent</u> difference in action of the CTXs may be related to differences in the concentration and type of free fatty acids in the two tissues in the absence of CTX (Fletcher and Lizzo, 1987), not differences in mode of CTX action.

One problem with determining the role of PLA_2 activity in the action of CTXs is that most investigators analyze PLA_2 contamination of CTX preparations by measuring the enzymatic activity on purified phospholipid or egg yolk substrates by various titration assays. Titration assays measure fatty acid release and cannot distinguish between PLA_1 (EC 3.1.1.32; removal of fatty acid from #1 position) and PLA_2 (removal of fatty acid from #2 position) activities. PLA_1 and PLA_2 activities are collectively referred to in the present study as PLA activity. The results of titration assays are considered to accurately reflect PLA_2 activity in venom research, as PLA_1 activity is not present at detectable levels in snake venoms (Rosenberg, 1979). CTX preparations that have no PLA activity on artificial substrates have been assumed to be enzymatically inactive on biological membrane systems such as

the red blood cell. Rosenberg (1979) has cautioned against such assumptions about PLA_2 activity and has suggested that PLA_2 activity must be directly determined on the biological substrate that is used for pharmacological or toxicological studies.

Snake venom CTX shares many of the properties of bee venom melittin, including interactions with PLA_2. The action of melittin on biological membranes has been better characterized than that of CTX, therefore melittin serves as a good model for CTX studies. Melittin, a low molecular weight (2,840) polypeptide of 26 amino acids, comprises about 50% of the dry weight of bee venom (Habermann, 1972). Among its toxic actions, melittin causes hemolysis of red blood cells and is cytolytic to other cell types (Habermann, 1972). Melittin enhances the rate of bee venom PLA_2 activity 5- to 6-fold on sonicated (Mollay and Kreil, 1974) and up to 300-fold on nonsonicated (Yunes et al., 1977) liposomes. Similar studies have not been conducted with CTX, despite its reported interaction with PLA_2. Understanding the mechanism by which melittin enhances bee venom PLA_2 activity is important, as melittin is used by investigators as a probe to activate tissue PLA_2 activity (Mollay et al., 1976; Shier, 1979). The use of melittin as a tissue PLA_2 activator evolved from the initial observations with bee venom and presumes some specificity to this action. Recently investigators have proposed that melittin enhances the rate of bee venom PLA_2 activity on multilamellar vesicles (nonsonicated liposomes) by converting these concentric bilayers into large unilamellar vesicles and thereby exposing a greater phospholipid surface to the enzyme (Dufourcq et al., 1986). However, these studies did not actually examine the interaction between melittin and PLA_2 and were conducted in the absence of Ca^{2+}, which, in addition to supporting PLA_2 activity, normally binds to the headgroup of phospholipids and stabilizes membranes. The absence of this divalent cation could have contributed to the destabilizing effect of melittin on the multilamellar vesicles. Also, the conversion of multilamellar vesicles to large unilamellar vesicles does not explain the enhancement of bee venom PLA_2 on unilamellar substrates (sonicated liposomes) by melittin (Mollay and Kreil, 1974). However, melittin appears to cause local perturbations of bilayers (Dufourcq et al., 1986) that may somehow increase the access of PLA_2 to the phospholipid substrate. As with CTX, it is difficult to isolate melittin fractions that are free from contamination with venom PLA_2 activity (Mollay et al., 1976).

The relationship between the potency of CTX and the lipid composition of the target membranes is unclear (Condrea, 1979). It has been suggested that tissues with higher levels of free fatty acids are less sensitive to the interaction between CTX and PLA_2 (Fletcher and Lizzo, 1987). The relationship between free fatty acids and the response of membranes to CTX alone has not been directly examined.

The induction of contractures in skeletal muscle very possibly reflects an increase in free myoplasmic Ca^{2+} concentration. This increased myoplasmic free Ca^{2+} concentration would promote prolonged actin/myosin interactions. Indeed, leakage to one or more ions is suspected following CTX exposure (Chang, 1979). Cytoplasmic free Ca^{2+} concentrations can be monitored in lymphocytes with the use of fluorescent indicators, such as indo-1/AM and fura-2/AM (Grynkiewicz et al., 1985).

Internalization is required for the action of a number of bacterial toxins (Middlebrook and Dorland, 1984). The rapid time to onset of effect (seconds; Fletcher and Lizzo, 1987) suggests signal transduction is a more

likely mechanism for CTX action. Regardless the actual location of the toxin molecule (inside or outside of the cell), it is important in developing prophylactic and therapeutic measures to determine accessibility of antibodies to the toxin site for neutralization.

Rationale:

Several model systems will be used to examine: (1) the mode of action of snake venom CTXs; (2) the dependence of this action on the membrane lipid composition; (3) the interaction of CTX with PLA activity; (4) the effects of membrane composition on CTX action; (5) internalization of CTX, and; (6) the similarities and differences between CTXs and the presynaptically-acting snake venom PLA_2s. The advantages and disadvantages of each system are:

1. Skeletal Muscle. Contractures of skeletal muscle are believed to reflect the "true" cardiotoxic mechanism of CTXs. A special advantage of this preparation is that we have access to human skeletal muscle, which differs from the commonly used rat diaphragm preparation in Ca^{2+} dependence of CTX action (Fletcher and Lizzo, 1987). Disadvantages of skeletal muscle are the great degree of biological variability involved in examining contracture induction, the small number of studies that can be run in a single day and the inability to conveniently use fluorescence probes to directly monitor myoplasmic Ca^{2+} concentrations or patch-clamp electrophysiology to directly examine ion currents.
2. Erythrocytes. Despite some disagreement (Harvey, 1985), the action on red blood cells may reflect the "true" cardiotoxic mechanism of CTXs (Fletcher and Lizzo, 1987). The red blood cell has many advantages, including: it is a simple model system in which all the lipids are contained in the plasma membrane (no organelles in erythrocytes); red blood cells of different lipid composition can be obtained from a wide variety of species (we have contacts with University of Pennsylvania School of Veterinary Medicine and the Philadelphia Zoo); the hemolysis assays with red blood cells allow many experiments to be conducted in a single day on the same batch of cells and they are far less subject to biological variability than skeletal muscle. The disadvantages of RBCs are the limited number of toxins that are active on them and their paucity of responses that can be examined, as apposed to excitable cells (muscle and nerve).
3. Lymphocytes. We have recently demonstrated lymphocytes to be a target for CTX action. Lymphocytes, like red blood cells, can be obtained from a large number of species. Patch-clamp electrophysiology for monitoring plasmalemmal ion currents and direct monitoring of cytoplasmic Ca^{2+} can be done with lymphocytes. Lymphocytes can be preloaded overnight with radiolabeled lipid precursors and subtle aspects of lipid metabolism examined the following day. Disadvantages of lymphocytes include their lack of availability (they must be isolated rapidly from fresh blood - free donors are scare) and limited number of ion currents (primarily K^+).
4. Platelets. Platelets are a rich source of tissue PLA_2 activity. Human platelets are readily available from the Red Cross.
5. Synaptosomes. Synaptosomes, or other nerve preparations, are essential for studies on presynaptically acting PLA_2 neurotoxins, as these

toxins are specific for nerve tissue. Synaptosomes have the advantages that that they provide a relatively large tissue mass for biochemical studies of presynaptic terminals impossible to obtain with phrenic nerve-diaphragm preparations, intrasynaptosomal Ca^{2+} can be monitored with fluorescent dyes and acetylcholine release can be monitored with radiolabeled choline. The major disadvantage of synaptosomes is that they are not currently a good model for PLA_2 toxin studies. The reasons that they are not appropriate are: they have greatly elevated fatty acid levels that may mask subtle effect of PLA_2 toxins, and; the optimum conditions for simulating the action of the toxins at the neuromuscular junction have not been satisfactorily worked out. We are attempting to correct these problems.

6. Cell Lines. Cell lines provide homogeneous biological material. They can be rich in ion currents, depending on the cell type. The epithelial cells, like lymphocytes, have an advantage over muscle in that intracellular cytosolic Ca^{2+} concentrations can be monitored directly with fluorescence probes, such as indo-1. Additionally, patch-clamp electrophysiological studies can be conducted in which the effect of the toxin on specific membrane currents can be directly examined. Skeletal muscle patch-clamp studies require the use of cell culture, which might suppress expression of toxin sensitive channels (appears to be the case in cultured human and equine skeletal muscle; unpublished observations). The advantage in using both cell types is that epithelial cells have Ca^{2+} and voltage activated K^+ channels and lymphocytes have K^+ channels inhibited by Ca^{2+}. Additionally, cell lines can be used for detailed anaysis of lipid metabolism, as the phospholipid and neutral lipid pools can be readily radiolabeled overnight. The major disadvantages of cell lines are the time spent cloning and maintaining the cells and the "abnormal" responses that may be induced by either culture conditions or the process of immortalizing the cells.

7. Artificial Membranes. Artificial membranes can be prepared in many different forms, all of which yield different types of information regarding membrane perturbing agents. The membranes can be: mixed micelles, which are monolayers of phospholipid and a detergent (usually Triton X-100); unilamellar vesicles, which are bilayers of either a single phospholipid or mixture of phospholipids, and; multilamellar vesicles, which are concentric bilayers of phospholipids. These different membranes, all having different amounts of phospholipid exposed to the incubation medium, can be used to examine substrate availability and membrane penetration in PLA_2 studies. The primary disadvantage of these preparations is their dissimilarity in behavior to more complex biological membranes.

To examine if possible differences in mechanisms of action may exist in this group of toxins and the role of PLA_2 activity in hemolysis, we compared the effects of red blood cell age, incubation temperature and pH, divalent cation type and concentration and addition of glucose on hemolysis induced by three cardiotoxins from different snake venoms to that induced by bee venom PLA_2. Similarities in hemolytic behavior observed between the CTXs in the present study would suggest that the same or very similar mechanism within this group of toxins induces hemolysis. The dependence of this mechanism on contaminating PLA_2 activity can be examined by modifying the histidine residue

at the catalytic site of contaminant PLA_2 with p-bromophenacyl bromide. The studies were conducted during the the first year of the grant and are included in the first Annual Report.

The contract also examines the utility of purified phospholipid substrate systems for predicting the PLA activity of CTXs on biological membranes. In addition, the relationship between hemolysis of red blood cells promoted by two typical snake venom CTXs and one abnormally large CTX is compared to the hydrolysis of membrane bound phospholipids by these toxins to determine if PLA activity is crucial to the mechanism of red blood cell hemolysis. Since Sr^{2+} is known to be as effective as Ca^{2+} in supporting CTX contractures in skeletal muscle (Fletcher et al., 1981; Fletcher and Lizzo, 1987) and yet less effective than Ca^{2+} in supporting PLA_2 activity (Iwanaga and Suzuki, 1979), the effects of these two divalent cations on red blood cell hemolysis and phospholipid hydrolysis induced by the CTXs are contrasted. The studies were primarily conducted during the the first year of the grant and are included in the first Annual Report. The studies were expanded on to some extent in the second year of the grant, especially as regards different forms of substrates (mixed micelles, unilamellar and multilamellar vesicle) and these are included in the present report.

The effects of the toxin on endogenous PLA_2 activity of the red blood cell and lymphocyte can be directly examined using radiolabeled phospholipids. In this system the enzyme activity of red blood cells (or lymphocytes) alone, CTX alone and red blood cells (or lymphocytes) plus CTX can be determined. Highly detailed studies of effects on endogenous PLA_2 can be conducted in cell lines with neutral and phospholipids preradiolabeled by feeding the cells ^{14}C-fatty acids overnight.

To develop potential prophylactic and therapeutic agents, pharmacological studies examining antagonists of CTX action are conducted. These studies include the PLA_2 inhibitor mepacrine (quinacrine), the partial antagonists of CTX-induced skeletal muscle contractures dantrolene (Fletcher and Lizzo, 1987) and the divalent cation, Mn^{2+}. Many of these studies were conducted in the first year and are included in the first Annual Report. A series of protein kinase C antagonists was also examined, as fatty acids (a product of lipolysis) activate protein kinase C (McPhail et al., 1984; Naor et al., 1988). Additionally pilot studies with a protein kinase inhibitor, sphingosine, suggested that protein kinase inhibition might antagonize CTX action. Cross-inhibition of protein kinase C by calmodulin antagonists also resulted in these inhibitors being examined. These inhibitors were examined as regards hemolysis of red blood cells by CTX.

Since PLA_2 activity enhances the action of snake venom CTXs and bee venom melittin (Condrea, 1979), the effects of exogenously added fatty acids on CTX-induced hemolysis should be examined. These are highly toxic products of PLA_2 activity that account for the synergy observed between this enzyme and halothane, which has many of the properties of CTX, and PLA_2 (Fletcher et al., 1987). Additionally, the synergism between PLA_2 and the difficulty in preparation of either CTX or melittin fractions that are free of venom PLA_2, make it essential to find a means to test the toxicity of CTX and melittin under conditions that considerably reduce this contaminating PLA_2 activity. Treatment of the toxin fractions with p-bromophenacyl bromide inactivates, or greatly reduces, PLA_2 activity, without affecting the basic mechanism of the toxin (Jiang et al., 1989).

Assuming that hemolysis may be the end result of a series of events

leading to leakiness of the cell, we sought to find a smaller molecule that might leak out long before hemoglobin. ^{3}H-Deoxyglucose is taken up by cells and phosphorylated to deoxyglucose-6-phosphate. Deoxyglucose-6-phosphate is then trapped within the cell cytoplasm. Therefore, nonspecific cell leakage would result in the release of ^{3}H-deoxyglucose-6-phosphate.

Since CTX is believed to increase ionic permeability (Chang, 1979), we chose to examine the effects of CTX on lymphocyte and epithelial cell line cytoplasmic free Ca^{2+}. Additionally, in collaboration with Dr. Steven Wieland (Dept. Anatomy, Hahnemann University), we examined plasmalemmal ionic currents in both cell types using the whole cell variant of patch-clamp recording.

Internalization of the toxin can be determined in a number of ways. Our initial studies examined the patterns of phospholipid hydrolysis and free fatty acid release. Later studies will examine the effectiveness of inhibitors of internalization and antibodies to CTX.

ß-bungarotoxin (ß-BTX) and other presynaptically-acting snake venom PLA_2s exhibit a triphasic action on acetylcholine release from the phrenic nerve-diaphragm preparation (Chang, 1979). The observation of these phenomena is based on electrophysiological studies of events (EPPs, MEPPs) having durations of milliseconds. The relative insensitivity of even the most sensitive biochemical assays for acetylcholine requires that the transmitter be collected over periods of seconds to minutes. Therefore, extensive analysis of the time and concentration dependence of ß-BTX action must be done in order to recreate in this biochemical model the same effects observed in the electrophysiological model. We have previously developed a crude method with which the second and third phases of the triphasic effect can be emulated in this biochemical model (Fletcher and Middlebrook, 1986). However, the model has to be refined, including application to a more highly purified synaptosomal fraction and improvment of the methods of isolation to reduce the extensive lipolysis that occurs during homogenization. Once this model has been sufficiently developed, then it can be applied to studies comparing the mechanisms of CTX action to those of the PLA_2 neurotoxins.

Experimental Methods:

Materials. Venom from Naja naja atra, CTX from Naja naja kaouthia venom (Lot# 125F-4007), bee venom PLA_2 (Apis mellifera), melittin, ß-bungarotoxin, Tris base, Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Mes (4-morpholineethanesulfonic acid), bovine serum albumin (fatty acid free), 2'-7'-dichlorofluorescein, free fatty acid (methylated and unmethylated) standards, phospholipid, triglyceride and cholesterol standards, and butylated hydroxytoluene were purchased from Sigma Chemical Company (St. Louis, MO). CTX from Naja naja atra venom was purified from venom by ionic exchange chromatography, as described in the first Annual Report. Venom from Bungarus fasciatus was a gift from Dr. T. Xue (Guangzhou Medical College, China). CTX from Bungarus fasciatus venom was purified, as previously described (Ji et al., 1983). The N. n. kaouthia and N. n. atra CTX fractions each appeared on SDS-PAGE slab gels as a single protein band of about 7,000 MW. The CTX from B. fasciatus venom appeared on the gels as a major band at approximately 16,000 MW and a minor band (<3% of the major band by scanning densitometry) at about 30,000 MW.

Hemolysis of Erythrocytes. Erythrocytes were obtained from the American Red Cross Blood Services and stored as CPD whole blood (AS-1) at 4°C. These

cells were used within about two weeks (plus or minus) of their expiration date.

Before incubation with toxin erythrocytes were washed three times in HEPES buffer (HEPES 20 mM, NaCl 130 mM, room temperature, pH adjusted to 7.4). Following the final centrifugation step 4 µl aliquots of packed red blood cells were added to 0.6 ml of the incubation medium (HEPES buffer with indicated pH, temperature, concentration of divalent cation and glucose). The preparations were incubated for 2 hr with or without toxin, centrifuged and hemoglobin release estimated by reading the absorbance of the supernatant at 540 nm. The 100% hemolysis point was determined by incubating 4 µl of erythrocytes in 0.6 ml of distilled water. Blanks containing no CTX were subtracted from all samples.

Treatment of Toxin Fractions With p-Bromophenacyl Bromide. Irreversible inactivation of PLA_2 activity was done by the method of Pieterson et al. (1974). Bee venom PLA_2 and N. n. kaouthia and B. fasciatus CTXs (30 µM) were incubated for 0 or 20 hr at 37°C in 600 µl of sodium cacodylate buffer (0.1 M) containing NaCl (0.1 M), p-bromophenacyl bromide (300 µM; added from 10 mM stock made up in acetone) and adjusted to pH 6.0. Following the reaction with p-bromophenacyl bromide, PLA_2 activity and hemolytic activity were determined. A mixed micelle substrate comprised of phosphatidylcholine from egg yolk (5 mM) and Triton X-100 (10 mM) was used to determine PLA_2 activity. The phospholipid was evaporated to dryness under N_2. Subsequently the detergent and 1 ml buffer [Tris (100 mM), bovine serum albumin (0.5%), and Ca^{2+} (2 mM), pH adjusted to 7.4] were added and the preparations were sonicated until clear. Treated PLA_2 or CTX (N. n. kaouthia and B. fasciatus CTXs were frozen and lyophilized first to concentrate the toxin) was added to the substrate buffer and the samples were incubated for 30 min at 37°C. The incubation was terminated by addition of extraction mixture and the free fatty acids were then titrated (Dole, 1956). Hemolytic activity was tested as described above using a 60 µl aliquot of the p-bromophenacyl bromide-treated toxin (3 µM final toxin concentration) and adjusting the hemolysis HEPES buffer (HEPES 20 mM, NaCl 130 mM, Ca^{2+} 2 mM) to pH 9.0 prior to addition of the toxin. The final pH of the actual incubation medium after mixing the cacodylate and HEPES buffers was 7.8.

Preparation of Mixed Micelle, Unilamellar and Multilamellar Substrates. Mixed micelles were prepared by evaporating the appropriate phospholipid substrate to dryness under N_2 and adding Triton X-100 at the indicated ratio to PC. Tris buffer (Tris 100 mM; Ca^{2+} 2 mM; BSA, 0.5%, pH 8.0) or HEPES buffer (HEPES 20 mM, Ca^{2+} 2 mM, BSA 0.5%, pH 7.4) was added and the preparations were sonicated until clear. In one case, radiolabeled substrate, L-3-PC (1-stearoyl-2-[1-^{14}C]arachidonyl), was added to this latter buffer at a concentration of 10 µM. Multilamellar vesicles were prepared by lyophilizing egg yolk PC overnight to remove all organic solvents. Tris buffer was added and the phospholipid (1 mM final concentration) was allowed to swell at room temperature for 2 hrs. Small unilamellar vesicles were prepared in the same manner as the multilamellar vesicles, except that the preparations were sonicated for 20 min before use.

Determination of Phospholipase A_2 Activity. The substrates above were incubated with or without PLA_2 or melittin for the indicated time and temperature. The incubation was terminated by extracting and titrating the free fatty acids (Dole, 1956), or, when using radiolabeled substrates, the incubates (1 ml) were extracted with 2 ml CH_3OH, and, after 30 min, 2 ml $CHCl_3$

and 1.5 ml H_2O were added. The lower phase (chloroform) was removed and evaporated under N_2 and brought up in 200 µl of $CHCl_3$ for spotting on silica gel plates. The neutral lipids were separated by 1-D TLC, as previously described (Fletcher et al., 1987). The plates were dried and the lanes scanned for radioactivity with a Raytest (McMurray, PA) RITA Radio TLC Imaging Analyzer. Free fatty acids were identified by comparing R_f values with radiolabeled standards developed in separate lanes on each TLC plate.

^{3}H-Deoxyglucose Release from Erythrocytes Induced by CTX. ^{3}H-d-Glu-6-p release from erythrocytes was determined as previously described (Jiang et al., 1987). Packed red blood cells were washed three times with N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)-buffered preloading solution (HEPES 20 mM, NaCl 130 mM pH 7.4) The packed erythrocytes pellets (2 ml) were added to 2 ml of preloading solution and then were incubated with ^{3}H-dGlu (5 µCi) for 3 hr at 37°C. The loaded cells were then washed three times with cold (4°C) preloading solution. Aliquots (4 µl) of preloaded cells were added to 200 µl of incubation solution (preloading solution containing 5 mM glucose and indicated divalent cation) and incubated at 37°C for the indicated time. The tubes were centrifuged and 200 µl supernatant was added to 3 ml of the complete counting cocktail (RPI; Prospect, IL). Radioactivity was determined with a Beckman scintillation counter (Model LS 5801). The 100% ^{3}H-dGlu-6-p release was determined by incubating 4 µl of erythrocytes in 200 µl of H_2O. Hemolysis of human erythrocytes was performed as previously described (Jiang et al., 1989) Before incubation with CTX, erythrocytes were washed three times (HEPES 20mM NaCl 130 mM pH 7.4). Following the final centrifugation, a 4 µl aliquot of packed red blood cells was added to 200 µl incubation medium (like the ^{3}H-dGlu-6-p release experiment) then incubated at 37°C for the indicated time, centrifuged and the hemoglobin release estimated by reading the absorbance of the supernatant at 540 nm. The 100% value for hemoglobin release was determined by adding 4 µl of erythrocytes to 200 µl distilled water.

Lymphocyte and Epithelial Cell Line Isolation and Intracellular Ca^{2+} Determination (Indo-1/AM). Whole venous blood was collected in heparinized tubes and lymphocytes processed within 2 hrs. Aliquots (3 ml) of whole blood were added to 5 ml buffer (NaCl 120 mM; Hepes 20 mM; KCl 3 mM; dextrose 10 mM; $CaCl_2$ 1 mM; pH 7.4; room temperature). Whole blood plus buffer (8 ml) was carefully layered on 3 ml Histopaque (Sigma Diagnostics, St. Louis, MO). The samples were centrifuged at 400 x g for 30 min at room temperature. The interface layer (mononuclear leukocytes, predominantly lymphocytes) was removed and washed twice at 300 x g for 10 min with RPMI-1640. Cultured epithelial cells were obtained from Dr. Leslie Krueger (Hahnemann University). Lymphocytes and epithelial cell lines were loaded with indo-1/AM using standard methodology (Grynkiewicz et al., 1985). Growth medium was removed from confluent epithelial cells in T-75 flasks. Lymphocytes or cell lines were resuspended in 2 ml RPMI-1640. indo-1/AM was added to a final concentration of 2 µM and the lymphocytes incubated for 30 min at 37°C protected from light. Lymphocytes were washed twice to remove free indo-1/AM (300 x g, 10 min) and resuspended (100 µl RPMI-1640/10 ml initial whole blood; 20 µl = 10^6 lymphocytes). The samples were equilibrated at 37°C for 15 min and CTX injected in a volume of <5% of the total incubation volume. Fluorescence was monitored using a Shimadzu RF-540 spectrofluorimeter. The excitation wavelength was 340 nm and the emission wavelength was 390 nm (Luckhoff, 1986). The maximum and minimum fluorescence values were determined by the successive

addition of ionomycin (1 µM) and Mn^{2+} (5 mM).

Membrane Currents Using the Whole Cell Variant of Patch Clamp Electrophysiology. Cells were voltage-clamped at room temperature (21-23°C) using the whole-cell variant of the patch clamp method previously described for HL-60 cells (Wieland et al., 1987). The resistence of pipettes was in the range of 1.5 to 2.5 x 10^6 ohms. The external medium was : NaCl 137 mM, KCl 5 mM, $CaCl_2$ 2 mM, glucose 5 mM and Hepes 10 mM, pH 7.3. The pipette medium was: potassium glutamate 105 mM, KF 25 mM, KCl 10 mM, EGTA 10 mM, Hepes 10 mM, pH 7.3. The data were analyzed using the pClamp (IBM) program.

Preparation of Synaptosomes and Determination of Acetylcholine (ACh Release). Early studies will be done using the crude P_2 pellet preparation, as previously described (Fletcher and Middlebrook, 1986). Once the methods of ACh release are worked out, we will use the more highly refined synaptosomal preparation described by Dunkley et al. (1988). Briefly, whole brain from rat or mouse is homogenized (1 g tissue per 9 ml) in a Teflon-glass homogenizer containing sucrose (0.32 M), EDTA (1 mM), dithiothreitol (0.25 mM), pH 7.4 (4°C). The homogenate is centrifuged (1,000 x g; 10 min) and the supernatant adjusted to 14 ml for a protein concentration of 5 mg/ml. The supernatant is applied to a discontinuous Percoll gradient (2 ml each 23%, 15%, 10%, 3% Percoll; v/v; pH 7.4) and centrifuged at 32,500 x g for 5 min. Fractions 3 and 4 will be used in our studies. Incubation of the synaptosomes with radiolabeled choline and β-BTX, centrifugation (microcentrifuge; 4°C) of the samples and removal of the supernatants is similar to previous studies (Fletcher and Middlebrook, 1986). ACh release is determined by lyophilizing the supernatant overnight. The sediment is resuspended in methanol (50 µl), spotted on TLC plates, developed in one dimension with n-butanol:methanol:acetic acid:ethylacetate:water (4:2:1:4:3; Bluth et al., 1980) and the radioactivity corresponding to ACh and choline analyzed by an imaging scanner.

Results:

PROBLEM 1. What are the effects of CTX alone and CTX-PLA_2 combinations on contracture induction of human and rat skeletal muscle and on Ca^{2+} transients in human and equine lymphocytes?

Patch-Clamp Electrophysiological Studies on the Action of a Phospholipase A_2 With CTX-Like Properties from B. fasciatus Venom in Lymphocytes and Cell Lines. We have begun collaborative studies with Dr. Steven Wieland (Dept. Anatomy, Hahnemann University) to examine the effects of the CTXs on membrane currents using patch-clamp electrophysiological approaches. Whole cell patch-clamp studies (gigaohm-seal) have revealed that B. fasciatus CTX (0.5-2 µM) enhances Ca^{2+}-activated K^+ channels in human epithelial cell lines (Figure 1), in contrast to the inhibition of Ca^{2+}-inhibited K^+ channels observed in lymphocytes reported in the last quarterly report. Unlike the transient and reproducible increase in cytoplasmic Ca^{2+}, the effect on the K^+ channels is irreversible. Most importantly, the toxin does not cause a leakage current suggestive of nonspecific membrane damage. Therefore, the interaction of CTX with the target cell is extremely complex, involving transiently affected Ca^{2+} currents and irreversibly modified K^+ currents. The toxin appears to lock on a Ca^{2+}-dependent process.

Effects of a Phospholipase A_2 With CTX-Like Properties from B. fasciatus

Venom on Ca^{2+} Regulation in Lymphocytes and Cell Lines. The changes in the voltage-dependent activation of K^+ currents of epithelial cells and peripheral lymphocytes caused by exposure to B. fasciatus CTX were strikingly similar to the changes caused by exposure to increased intracellular Ca^{2+}. Therefore, we directly tested the hypothesis that B. fasciatus toxin produced elevated intracellular Ca^{2+} in epithelial cells and in lymphocytes by exposing a suspension of indo-1 loaded cells to the toxin. Exposure of either cell type to 1 μM toxin showed transient elevation on intracellular Ca^{2+} which began immediately after addition of the toxin (Figure 2). With 5 different epithelial cell cultures, the mean free resting Ca^{2+} level was 60±16 nM (mean±SEM). The peak increase in intracellular free Ca^{2+} was 70±27 nM above resting level. The time to peak Ca^{2+} concentration was 23±2 sec and the time to return to baseline level was 109±20 sec. The resting free intracellular Ca^{2+} level in lymphocytes from 3 subjects was 150±23 nM; the peak increase in free Ca^{2+} was 48±11 nM above resting level. The time to peak Ca^{2+} concentration was 206±9 sec, and the time to return to 50% of baseline level was 251±5 sec. In epithelial cells the return to baseline was much faster than in lymphocytes. However, in both cell types intracellular Ca^{2+} level perturbations appeared shorter-lived and of lesser magnitude than the pipette concentrations which caused similar effects on K^+-channel function.

PROBLEM 2. What is the dependence of CTX action on fatty acid distribution and the free fatty acid content of muscle and red blood cells?

No studies specifically related to this problem were conducted this year.

PROBLEM 3. What are the hemolytic effects of CTX alone and CTX-PLA_2 combinations in red blood cells from different species?

Kinetics of Hemolysis by the Snake Venom CTXs. We have begun an analysis of the kinetics of hemolysis by the snake venom CTXs. We previously reported that aging red blood cells results in greater hemolytic activity of the CTXs (see Annual Report 1987-1988). This increased susceptibility of red blood cells to lysis by the CTXs is the result of an increase in V_{max} for the first 4 days, but a decrease in K_m in addition to the increase in V_{max} after 6 days (Figure 3). Changing pH greatly increases the hemolytic action of CTX (see Annual Report 1987-1988). This shift can be attributed to both an increase in V_{max} and K_m (Figure 4). In the presence of Ca^{2+} there appears to be a break in the dose-response curve above about a 3 μM concentration of CTX. This break can be attributed to an increase in both K_m and V_{max} (Figures 5 and 6). In a Ca^{2+} medium the K_m is about 3 μM at low concentrations of CTX (Figure 6A). This is almost 10-fold higher than observed in a Ca^{2+}-free medium (0.45 μM; Figure 7), in agreement with Ca^{2+} competing with CTX for binding. The K_m values for Sr^{2+} and Ba^{2+} are about 1 μM and 1.25 μM, respectively (Figure 7), suggesting that these ions may compete with CTX for binding, but are not as effective antagonists as Ca^{2+}.

Effects of Protein Kinase C Inhibitors on Hemolysis by Snake Venom CTXs. Since our previously reported findings of altered K^+ channel activity by B. fasciatus CTX suggested irreversible activation of a Ca^{2+}-dependent pathway as a mechanism of CTX action, series of inhibitors of protein kinase C and calmodulin were examined as regards the hemolytic action of CTXs. Sphingosine (Figure 8) and polymyxin B (Figure 9), two protein kinase C inhibitors

(Merrill and Stevens, 1989; Mazzei et al., 1982; respectively) both inhibited hemolysis by the N. n. kaouthia CTX at low μM concentrations of antagonist. The calmodulin antagonists, such as trifluoperazine and chlorpromazine, are also reported to inhibit protein kinase C (DeRiemer et al., 1985). However, these agents (Figures 10 and 11) and phloretin (Figure 12), an additional nonspecific inhibitor of protein kinase C (Tanaka et al., 1986), were ineffective as antagonists of CTX-induced hemolysis. The most potent and specific inhibitor of protein kinase C, staurosporin (Tamaoki et al., 1986), was also ineffective as an antagonist of CTX action (Figure 13). These results suggest several possibilities, including: (1) only sphingosine and polymyxin B are effectively taken up by red blood cells and able to inhibit RBC protein kinase C; (2) the specific site of action of sphingosine and polymyxin B on the protein kinase C molecule is required for antagonism of CTX action, and; (3) the two effective antagonists of CTX action may be acting by a mechanism independent of protein kinase C. Examples of this lattermost possibility are: competition with CTX for the RBC binding site and binding of CTX to sphingosine or polymyxin B to effectively reduce the "active" concentration of CTX in the bathing medium. We next examined the kinetics of hemolysis using Lineweaver-Burke analysis. Both sphingosine and polymyxin B appear to be competitive inhibitors of hemolysis by the N. n. kaouthia CTX (Figure 14). Such a competitive inhibition could be explained by the three possible modes of action presented above.

^{3}H-Deoxyglucose (^{3}H-dGlu-6-p) Release from Erythrocytes Induced by CTX - Comparison With Hemolysis. The two CTXs and the PLA2 with CTX-like properties differ in hemolytic potency [N. n. kaouthia CTX > N. n. atra CTX >> B. fasciatus PLA2 (Jiang et al., 1989)]. We examined whether the three toxins exhibit a similar order of potency in eliciting the release of a considerably smaller molecule (^{3}H-dGlu-6-p). Indeed, the order of potency for ^{3}H-dGlu-6-p release from human red blood cells was N. n. kaouthia (Figure 15A) > N. n. atra (Figure 15B) >> B. fasciatus toxin (Figure 15C). The same order of potency was observed in Ca^{2+}-containing or Ca^{2+}-free media (Figure 15). Extracellular Ca^{2+} (2 mM) enhanced the hemolytic (Jiang et al., 1989) and ^{3}H-dGlu-6-p releasing (Figure 15) activities of N. n. kaouthia and N. n. atra CTXs, but only at concentrations of CTX $\geq$ 10 μM. These effects of Ca^{2+} were less evident for the weakly hemolytic B. fasciatus PLA2 with CTX-like properties. We compared the effects of five different divalent cation conditions (none, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}) on hemolysis and ^{3}H-dGlu-6-p release at two concentrations of N. n. kaouthia CTX (Figure 16). At a lower concentration (3 μM) of N. n. kaouthia CTX both ^{3}H-dGlu-6-p release and hemolysis in Ca^{2+}, Sr^{2+} or Ba^{2+} medium were similar, or only slightly lower, than in divalent cation free medium. However, at a higher concentration (10 μM) of N. n. kaouthia CTX both ^{3}H-dGlu-6-p release and hemolysis were greatly enhanced in a Ca^{2+} containing medium, but not in Sr^{2+} or Ba^{2+} containing media (Figure 16). Both ^{3}H-dGlu-6-p release and hemolysis were almost completely inhibited in Mn^{2+} (2 mM) containing medium at either 3 or 10 μM N. n. kaouthia CTX (Figure 16). To avoid the general stimulatory effect of Ca^{2+} at higher concentrations of CTX (Figure 15), the effects of varying the concentration of Ca^{2+} on ^{3}H-dGlu-6-p release and hemolysis were examined at a fixed N. n. kaouthia CTX concentration of 3 μM. Low concentrations of Ca^{2+} (<1 mM) enhanced ^{3}H-dGlu-6-p release and, to a greater extent, hemolysis relative to that in cation free media (Figure 17). Beyond a Ca^{2+} concentration of 2 mM, both ^{3}H-dGlu-6-p release and hemolysis were inhibited by Ca^{2+} in a dose-

dependent manner (Figure 17). As with hemolysis (Jiang et al., 1989), ^{3}H-dGlu-6-p release induced by N. n. kaouthia CTX (3 μM) exhibits a sharp increase in the range of pH 7.0 to 8.5 (Figure 18). We compared the time course of leakage of this large molecule to that of a considerably smaller molecule (^{3}H-dGlu-6-p) to determine if ^{3}H-dGlu-6-p release is a more sensitive indicator of CTX action. While ^{3}H-dGlu-6-p release appeared to be detectable at a slightly earlier time than hemolysis, there were no dramatic differences in the time courses of release of these two markers of CTX action (Figure 19).

In order to examine the possible role of trace contamination of the toxin fraction with PLA_2 in the hemolytic action of the N. n. kaouthia CTX, we treated the toxin fraction with p-bromophenacyl bromide (p-BPB), as previously described (Jiang et al., 1989). This treatment with p-BPB abolished the stimulatory effect of Ca^{2+} at higher concentrations of N. n. kaouthia CTX, making the toxin dose-hemolysis curves (e.g., see Jiang et al., 1989; Figure 2) identical whether done in Ca^{2+}-containing or Ca^{2+}-free media (data are not shown).

Substrate Specificities of Melittin and Bee Venom Phospholipase A_2. On mixed micelle substrates PLA_2 activity from bee venom exhibited a substrate specificity that favored polyunsaturated fatty acids at the #2 position (Figure 20). The activity on PC with saturated fatty acids at position 1 and 2 (dipalmitoyl PC) was lowest. Egg yolk PC, which is a mixed molecular species of substrate primarily comprised of 16:0, 18:1 (R_1, R_2) and 16:0, 18:2 (Porter et al., 1979), was next in preference and a pure unsaturated (#2 position) substrate was the most preferred. The PLA_2 activity in melittin (about 0.06% of the purified bee venom PLA_2) was most likely contamination with bee venom PLA_2, based on the low activity and similar substrate preference. Treatment of the melittin fraction with p-BPB reduced the enzyme activity to undetectable levels using the titration assay (eg., see Figure 24).

Hemolysis of Erythrocytes by Native and p-BPB-Treated Melittin and the Interaction of These Agents With Phospholipase A_2. Our initial studies (Figure 21) using mixed micelle substrates and fatty acid titration (Dole, 1956) assays have not supported the direct activation of venom PLA_2 by melittin, as suggested to occur by other investigators (Mollay and Kreil, 1974; Mollay et al., 1976). Indeed inactivation of PLA_2 histidine residues with p-bromophenacyl bromide (p-BPB) (melittin has no histidine groups) inhibits PLA_2 activity and totally abolishes any additive fatty acid release by the combination of melittin with PLA_2 *on mixed micelle substrates in the absence of added* Ca^{2+} (Figure 21). These results support our hypothesis that melittin does not specifically activate endogenous PLA_2 activity, but rather contains contaminating PLA_2 enzyme and/or modifies the phospholipid substrate. It is essential to verify that modification of contaminant PLA_2 in the CTX fraction with p-BPB does not also alter hemolytic activity of melittin, as this may reflect an altered ability to "activate endogenous PLA_2". In the absence of Ca^{2+} (by addition of EDTA) bee venom PLA_2 is inactive as a lipolytic enzyme. Additionally, EDTA antagonizes any hemolytic interaction between bee venom PLA_2 and native melittin (Figure 22A). Under these conditions (EDTA), the p-BPB-treated melittin retains hemolytic activity and the hemolytic activity is not enhanced by bee venom PLA_2 (Figure 22A). The p-BPB-treated melittin, in the presence of Ca^{2+}, is still able to interact with bee venom PLA_2 in a synergistic manner similar to nontreated melittin, as regards hemolysis (Figure 22B). We next examined the interaction between melittin and bee venom PLA_2 on more complex substrates, including unilameller vesicles (ULVs) and

multilameller vesicles (MLVs). Other investigators have reported an increase in *rate* of hydrolysis of ULVs (Mollay and Kreil, 1974) and MLVs (Yunes et al., 1977) and still other investigators (Dufourcq et al., 1986) have implied that this relates to the *extent* of hydrolysis on these substrates. We directly examined the extent of hydrolysis on ULVs and MLVs. The original studies on unilamellar vesicles were conducted at room temperature (Mollay and Kreil, 1974). Since the other experiments in the present study were carried out at 37°C, we compared the effects of melittin on PLA_2 activity at both temperatures to confirm the original finding and to directly compare these effects of melittin to those on the other substrates. Bee venom PLA_2 activity on unilamellar vesicles was enhanced by both the native melittin and p-BPB-treated melittin at either 20°C (Figure 23A) or 37°C (Figure 23B). In these experiments the melittin-induced enhancement of phospholipid hydrolysis by bee venom PLA_2 was related more to increasing the extent of hydrolysis, rather than enzyme activity *per se*, as 100% of the substrate was hydrolyzed in the presence of melittin. While the enzyme activities were slightly greater at 37°C, the interaction between melittin and bee venom PLA_2 was similar at either temperature. The action of bee venom PLA_2 was also enhanced by both the native and p-BPB-treated melittin fractions on mutilamellar vesicles at 37°C; however, much higher concentrations of both PLA_2 and melittin were required for even lower levels of phospholipid hydrolysis, presumedly due to the lesser substrate availability (Figure 23C). The PLA_2 contamination in the native melittin fraction (about 30 nM; based on relative activities in Figure 20) was apparently greatly enhanced by the presence of melittin. The absence of PLA_2 activity in the p-BPB-treated melittin fraction did not affect the enhancement of the much higher concentration (300 µM) of added bee venom enzyme (Figure 23C).

It is unclear from Figure 23 whether maximum substrate hydrolysis had occurred. Also, the presence of BSA may destabilize the membrane by removing the products of phospholipid hydrolysis. Therefore, the extent of hydrolysis by bee venom PLA_2 in the absence and presence of melittin in a BSA-free medium was examined at three time points (30, 60 120 min) to verify that a plateau of phospholipid hydrolysis had been reached (data not shown). Since the results in Figure 23 suggest that native and p-BPB melittin interact similarly, only the native melittin was used in this study. Bee venom PLA_2 activity on mixed micelles at a fixed concentration of PLA_2 and increasing concentrations of melittin is equal to the sum of the bee venom PLA_2 activity and the PLA_2 activity contamination in the melittin fraction (Figure 21). In addition, no increase in PLA_2 activity was observed upon adding p-BPB-treated melittin (30 µM) to bee venom PLA_2 (Figure 21). However, these studies were conducted in *the absence of* Ca^{2+}.

The substrate used in Figure 21 (egg yolk PC) contains primarily 18:1 and 18:2 at the #2 position of the glycerol moeity. It is possible that the activity of the bee venom PLA_2 enzyme might be specifically enhanced toward substrates containing highly unsaturated fatty acids, such as arachidonic acid (20:4). Therefore, we examined the effects of melittin on low concentrations of an arachidonic acid containing substrate embedded in an egg yolk PC:Triton X-100 matrix. Radiolabeled L-3-PC (1-stearoyl-2-[1-^{14}C]arachidonyl) was >90% hydrolyzed at higher PLA_2 concentrations (10 nM; data are not shown), suggesting complete access of this substrate to the enzyme when in miceller form. The lack of stimulating effect of melittin observed on egg yolk PC was also evident on this highly polyunsaturated fatty

acid-containing substrate (Figure 24).

The addition of EDTA completely inhibited any PLA_2 activity under all conditions (ULVs, MLVs, mixed micelles; data not shown). The mechanism underlying melittin-induced enhancement of ULV hydrolysis by bee venom PLA_2 is unclear. However, it has been suggested that melittin enhances PLA_2 activity on MLVs by converting MLVs to ULVs, which then makes the substrate more accessible to the enzyme. However these studies were conducted in an EDTA-containing medium (Dufourcq et al., 1986). We tested the effects of added Ca^{2+} (2 mM) on the interaction between melittin and bee venom PLA_2 in MLVs, ULVs and mixed micelles. Addition of Ca^{2+} (2 mM) was *essential* for melittin to enhance the activity of bee venom PLA_2 on MLVs and mixed micelles, *but not on ULVs* (Figure 25). The presence or removal of products of lipase activity might be essential for the interaction between melittin and bee venom PLA_2. Therefore, we examined the effects of BSA, which would remove the lytic products from the membrane. BSA slightly increased PLA_2 activity under all conditions, but had very little effect on the interaction between melittin and bee venom PLA_2 (Figure 26). It seems clear from these studies that melittin increases the accessibility of the substrate to the enzyme.

One puzzle that remained was how melittin could increase bee venom PLA_2 activity in a mixed micelle system, since all the substrate should be available to the enzyme in this system. It is well known that detergents increase hydrolysis of phospholipids by converting bilayers to mixed micelles and/or by providing a better matrix in which to hydrolyze monomers of phospholipids. We compared the effects of a commonly used detergent (Triton X-100) and melittin in enhancing PLA_2 activity (Figure 27). Melittin is effective in enhancing PLA_2 activity under conditions in which Triton is not, suggesting that melittin is not simply acting as a detergent.

In conclusion, while PLA_2 activity on MLVs might appear to be enhanced by melittin to a greater extent than that on ULVs when the *rate* of activity is determined, there is relatively little difference in the *extent* of hydrolysis between these substrates. The major difference between sonicated (ULV) and unsonicated (MLV) substrates was the enhancing action of melittin even in low Ca^{2+} containing medium in the case of the former (Figure 25).

Comparison of Snake Venom CTX and Melittin Mechanisms. Snake venom CTX has also been suggested to activate endogenous tissue PLA_2 activity (Teng et al., 1984), making the melittin studies very relevant to the present grant. We therefore compared the Ca^{2+} dependence of the hemolytic activities of melittin and the N. n. kaouthia CTX to determine indirectly if sufficient PLA_2 contamination exists in these fractions to enhance the hemolytic activities of either melittin or N. n. kaouthia CTX. The addition of EDTA to inhibit any PLA_2 activity that might be a contaminant of the toxin fractions resulted in a significant decrease in hemolytic activity only for native melittin (Figure 28). Hemolysis by the p-BPB-treated melittin was relatively unaffected by the presence of EDTA. The hemolytic activity of the N. n. kaouthia CTX fraction was slightly increased by EDTA (Figure 28), suggesting no involvement of trace PLA_2 contamination in hemolysis by this toxin at this low concentration. To examine if melittin and N. n. kaouthia CTX share a similar mechanism of hemolysis, the two were examined in combination with one another. The N. n. kaouthia CTX is a less effective hemolytic agent than melittin. Therefore, we expected the N. n. kaouthia CTX to act as a partial agonist and actually antagonize the hemolytic action of melittin if both agents act through the same mechanism. In contrast, melittin and CTX when added together were more

potent and exerted a greater degree of hemolysis than melittin alone (Figure 29). Additionally, Mn^{2+} was not as effective an antagonist of melittin action (Figure 29) as has been demonstrated for N. n. kaouthia CTX (Figure 16).

PROBLEM 4. What are the effects of CTX on endogenous lipolytic enzymes in skeletal muscle and red blood cells?

No studies specifically related to this problem were conducted this year.

PROBLEM 5. What is the role of toxin internalization in the action of CTX?

No studies specifically related to this problem were conducted this year.

PROBLEM 6. What are the similarities and differences between CTXs and the presynaptically-acting snake venom PLA_2s?

Studies on the Pre-Synaptically-Acting Snake Venom Phospholipases A_2. This is a newly added goal. We have purified two presynaptically-acting snake venom neurotoxins from Agkistrodon halys pallas (Figure 30). These toxins, designated peaks 8 and 9, are about 15,000 and 16,000 MW, respectively, based on PAGE. Both peaks have phospholipase A_2 activity, with peak 8 about 3-4 x more active than peak 9. The LD_{50} for peaks 8 and 9 are 270 µg/kg and 8 mg/kg. Previous investigators have only reported a single toxin with a pI of 6.9. We have been determining which synaptosomal preparations would be the most suitable for use in these studies. We have settled on a rapid procedure that has high yields of viable synaptosomes (Dunkley et al., 1988). We have isolated one batch of synaptosomes, but had no success in loading them with indo-1/AM. We will attempt to load these synaptosomes with fura-2/AM, as several cells and organelles are not able to load indo. We anticipate both quin-2 (Ashley et al., 1984; Komulainen and Bondy, 1987) and fura-2 (Komulainen and Bondy, 1987) will work, based on previous published data. Using synaptosomes, we have begun to examine the effects of presynaptically-acting PLA_2 toxins on acetylcholine (ACh) release from synaptosomes. Since most of our experience has been with a P_2 fraction (Fletcher and Middlebrook, 1986), we have used this approach to develop the technique for ACh analysis. Later, when we are convinced that the ACh analysis works correctly, we will change to a considerably improved method for synaptosome isolation (Dunkley et al. 1988). A radiogram of the radioactivity of a single lane on a TLC plate developed in one-dimension with the solvent system (Bluth et al., 1980) is shown in Figure 31. In this case the effects of ß-bungarotoxin (0.5 µM) were examined on ACh release in a P_2 fraction of murine synaptosomes. There was very little ACh released from untreated preparations (data not shown). When the Ca^{2+} ionophore A23187 (10 µM) was used an increase in ACh *and* choline resulted (Figure 32). Developing a system monitoring both ACh and choline with such clear separation allows nonspecific lysis (related to choline leakage) to be monitored. Pilot studies suggest that synaptosomes from mouse brains are more sensitive to ß-bungarotoxin than are synaptosomes from rat brains (data not shown). Concurrent with development of ACH analysis, we are investigating the effectiveness of various agents in inhibiting lipolytic activity during preparation of synaptosomes. In addition to a high background of free fatty acids and lysophospholipids (about 10-fold higher than normal) lipolytic activity during homogenization can result in altered Ca^{2+} binding (Walters et

al., 1986). Nerve gases are effective inhibitors of lipases (Walters et al., 1986), but the use of these agents is undesirable as they are highly toxic and irreversible. We have tested other less toxic and reversible cholinesterase inhibitors (physostigmine and neostigmine) and have found them to be ineffective lipase inhibitors. Pilot studies have suggested that the less toxic, but irreversible lipase inhibitor p-bromophenacyl bromide may be a useful agent in synaptosome isolation.

Discussion and Conclusions:

Mode of CTX Action in Cell Lines and Lymphocytes. The PLA_2 with CTX-like properties from B. fasciatus venom irreversibly alters the response of Ca^{2+}-regulated K^+ channels, such that Ca^{2+}-activated channels (lymphocytes) are activated by the toxin and Ca^{2+}-inhibited channels (epithelial cell lines) are inhibited by the toxin. Therefore, this toxin appears to mimic increased levels of Ca^{2+} within the cytoplasm. Pilot studies with the cobra CTX from N. n. kaouthia venom did not demonstrate a similar action on K^+ channels (data not shown). However, too few studies were performed to be conclusive. In contrast to the simple conclusion that B. fasciatus CTX increases cytoplasmic Ca^{2+}, the toxin only transiently elevates Ca^{2+} levels in both cell types. Therefore, the toxin appears to "lock on" a Ca^{2+}-dependent process regulating K^+ channels. An example of a process that might be activated ("locked on") would be a protein kinase. Since the presynaptically-acting PLA_2 neurotoxins also alter K^+ channel function (Dryer and Penner, 1987), it is tempting to speculate that these two types of toxins may have a common mechanism, but act at different target sites.

Effects of Divalent Cations on CTX Action. Divalent cations have varied effects on snake venom CTX action that are dependent on the concentration of toxin and the type and concentration of divalent cation. These effects of divalent cations are the same on CTX action whether hemoglobin or ^{3}H-dGlu-6-p release is examined. At low concentrations of toxin ($\leq$ 3 µM), divalent cations have only a slight stimulatory effect at concentrations < 2 mM and dramatic dose-response related inhibitory effects > 2 mM. However, at higher ($\geq$ 10 µM) concentrations of toxin, a very different effect of divalent cations was observed. At these higher concentrations of toxin, only Ca^{2+} (2 mM) stimulated hemolysis or ^{3}H-dGlu-6-p release, not Sr^{2+} or Ba^{2+}. Mn^{2+} was a potent inhibitor of CTX action at low and high concentrations of toxin. This observation is in agreement with a previous study in a rat atrial preparation in which the depolarizing and action potential blocking effects of a PLA_2 with CTX-like activity were antagonized and reversed by Mn^{2+} (Fletcher et al., 1982). Thus, understanding the mechanism of Mn^{2+} action might be important in elucidating the action of the cobra venom CTXs.

Significance of Contamination of CTX Fractions With Venom PLA_2. CTX and PLA_2 act synergistically to induced hemolysis of red blood cells (Condrea, 1974; 1979). It is difficult to purify CTX fractions that are free from PLA_2 contamination (Louw and Visser, 1978). Snake venom PLA_2 is a Ca^{2+}-dependent enzyme (Iwanaga and Suzuki, 1979). Therefore, it is possible that the enhanced hemolytic activity of high concentrations of the CTXs in Ca^{2+} medium is due to activation by Ca^{2+} of trace amounts of PLA_2 contaminating the toxin fraction. We have previously reported that our N. n. kaouthia and N. n. atra CTX fractions are relatively free of PLA_2 contamination (<0.0001%) as determined

by titration methods (Dole, 1956) using mixed micelle substrates of Triton X-100 and egg yolk phosphatidylcholine (Jiang et al., 1989). Using more sensitive approaches, such as gas chromatographic analysis of free fatty acids produced by the CTX fractions in biological membranes (erythrocytes), or PLA_2 activity of the CTX fractions on radiolabeled substrates (phosphatidylcholine; ^{14}C-ß-arachidonyl), we have observed very low levels of PLA_2 activity in these fractions (unpublished observations). The trace PLA_2 activity on radiolabeled substrates is reduced by about 95% following treatment of the CTX fraction with p-BPB (unpublished observations), suggesting that it is due to contamination with venom PLA_2 and is not intrinsic to the CTX, which lacks the histidine residue reacting with p-BPB (Naja naja siamensis; Dimari et al., 1975). This p-BPB treatment has no effect on the hemolytic activity of the N. n. kaouthia CTX at low concentrations (3 µM) in a Ca^{2+} containing medium (Jiang et al., 1989). Therefore, it is unlikely that this low level of contamination of the CTX fractions with PLA_2 has any relationship to hemolytic activity or ^3H-dGlu-6-p release at low concentrations of CTX. However, at higher concentrations of N. n. kaouthia CTX (30 µM) the stimulatory effect of Ca^{2+} on hemolysis is abolished with p-BPB treatment. These latter results highly suggest that trace PLA_2 contamination of the CTX fraction is involved in this stimulatory effect of Ca^{2+} through activation of the Ca^{2+}-dependent enzyme activity. It is unlikely that the antagonistic effect of Mn^{2+} on hemolytic activity is due to inhibition of trace PLA_2 activity, as Mn^{2+} is an effective antagonist of CTX action at concentrations of toxin (3 µM) that do not exhibit hemolytic activity stimulated by PLA_2 contamination.

Endpoint of CTX Action - Lysis of a Subpopulation of Cells vs. Opening of Membrane Pores. The relatively low levels of either ^3H-dGlu-6-p or hemoglobin release suggest that either: 1) only a subpopulation of cells is affected by the toxin and this is an all-or-none phenomenon, or; 2) all of the cells exposed to the toxin undergo only partial lysis. The similarities between ^3H-dGlu-6-p and hemoglobin release, however, suggest that the second possibility is unlikely. If all the cells underwent partial lysis (slight opening of the leakage "pores"), then ^3H-dGlu-6-p release would exceed hemoglobin release, as the smaller marker would be released at much greater amount than the larger marker. What population of cells would most likely be susceptible to lysis? Other investigators have reported that young red blood cells are resistant to hemolysis relative to aged cells when both populations are isolated from fresh human red blood cells (Chen et al., 1984). In vitro aging of red blood cells also leads to considerably greater levels of hemolysis by CTXs (Jiang et al., 1989). Therefore, the older blood cell population is most likely the subpopulation susceptible to lysis by CTXs.

Effects of Erythrocyte Age, Incubation pH, Toxin Concentration and Inhibitors of CTX Action on the Kinetics of Hemolysis. Several conditions alter CTX action by affecting either the K_m or V_{max} of toxin action, or both. In the simplest terms, the V_{max} for CTX action probably reflects the accessibility of the target sites to the enzyme; whereas, the K_m reflects the binding affinity to the exposed target sites. An alternate, and less likely in this case, explanation for the V_{max} is that binding to the same number of sites results in a greater net effect of the toxin. As red blood cells age, the V_{max} first increases (up to 4 days) with no change in K_m. At six days of aging the K_m decreases, meaning that the toxin not only has greater access to the target sites, but the binding affinity is also increased. In contrast, a higher pH of the incubation medium is reflected in increased V_{max} (greater

number of exposed sites), but also an increased K_m (decreased binding affinity). The net effect of increasing both V_{max} and K_m is greater hemolysis at any one concentration of toxin and an increased requirement for toxin for the new (much higher) maximum effect. The increase in K_m and V_{max} at high (> 3 μM) concentrations of CTX *and in the presence of* Ca^{2+} most likely reflect the increased number of binding sites through the action of the PLA_2 activity contaminating the toxin fraction. The shift in K_m may relate to the degree of contamination with PLA_2; that is, at least 3 μM CTX is required for enough PLA_2 contamination to affect the assay. Therefore, the K_m will be shifted to > 3 μM to begin with. Divalent cations appear to exhibit a competitive inhibition of CTX action. Ca^{2+} increases the K_m at low concentrations of CTX, with little effect on V_{max}. Likewise, Sr^{2+} and Ba^{2+} increase the K_m, but not to the same extent as Ca^{2+}. These effects are visualized most clearly in Figure 16A.

The two effective inhibitors of CTX action (sphingosine and polymyxin B) also appear to be competitive inhibitors, as the K_m is the most affected kinetic parameter. Whether this site of competition is the same as the site that divalent cations interact with is unclear.

Melittin/PLA_2 Interactions - Model for CTX Action. As previously mentioned, CTX and melittin share similar properties, including hemolysis of red blood cells and acting synergistically with PLA_2. Both toxins have been used as probes to activate tissue PLA_2 activity. Much more is understood about melittin action on membranes than about CTX action. Prior to conducting the same studies on CTX that have previously been used to elucidate the mechanism of melittin action, it was crucial that we ensure that we achieved the same results with melittin as reported in the literature. The present study examined the interaction action between melittin and bee venom PLA_2. The maximum extent of hydrolysis of the substrate, not enzyme activity *per se* was studied on a variety of forms of substrate, including multilamellar vesicles, unilamellar vesicles and mixed micelles to test if melittin increases the activity of the PLA_2 enzyme by simply increasing the substrate availability. To avoid the confounding results obtained with trace PLA_2 contamination, melittin fractions were treated with p-BPB to inhibit PLA_2 activity. This treatment did not affect the hemolytic activity of melittin, but greatly reduced PLA_2 activity in the melittin fraction and, therefore, allowed the use of Ca^{2+} in the bathing medium.

Bee venom PLA_2 is incapable of penetrating either natural (Zwaal et al., 1975; Fletcher et al., 1987) or artificial (Sundler et al., 1978; Wilschut et al., 1979) bilayers. The predicted order of extent of substrate hydrolysis by this enzyme based on this simple fact is mixed micelle > unilamellar vesicles > multilamellar vesicles. The stimulation of PLA_2 activity by melittin would be predicted to be related inversely to the amount of substrate available to the enzyme. The multilamellar vesicles have the least substrate available and are the least readily hydrolyzed by the PLA_2 enzyme in the absence of melittin. If melittin simply formed a complex with phospholipids that makes them more susceptible to attack, then hydrolysis of mixed micelles would also be enhanced by the toxin. However, we observed no enhancement by melittin of the rate of PLA_2 catalyzed hydrolysis of either the egg yolk PC, or arachidonic acid-containing molecular species of PC.

While the formation of unilamellar vesicles from multilamellar vesicles (Dufourcq et al., 1986) might at least partially explain the activation of PLA_2 activity by melittin, the subsequent action of melittin on the bilayer is

less clear. The effects of melittin on artificial substrates are complex and extrapolation of these results to biological substrates is difficult. PC bilayers can be converted to micelles (Dufourcq et al., 1986), whereas, cardiolipin (Batenburg et al., 1987a) and other negatively charged phospholipid (Batenburg et al., 1987b) vesicles can be converted to the H_{II} conformation. In contrast, melittin inhibits the natural H_{II} phase formation typical of phosphatidylethanolamine and stabilizes bilayer formation (Batenburg et al., 1988). Dipalmitoyl PC and distearoyl PC mixtures do not exhibit phase separation on addition of melittin; however, addition of dipalmitoylphophatidylglycerol to either of these phospholipids does result in phase separation (Lafleur et al., 1989). Formation of micelles (Dufourcq et al., 1986) at small sites on the membrane does not fully explain the complete hydrolysis of the substrates observed in the present study. However, it is possible that hydrolysis of a region around a discoidal partical could allow penetration of the PLA_2 enzyme into the interior of the bilayer and then allow complete hydrolysis of the interior phospholipids.

The present study demonstrates that melittin does not at all enhance the action of bee venom PLA_2 on mixed micelle substrates in the absence of Ca^{2+}, but does increase bee venom PLA_2 activity on mixed micelles in the presence of Ca^{2+}. Indeed, in the absence of added Ca^{2+}, the only increase in PLA_2 activity could be attributed to the small amount of contamination (< 0.1%) of the melittin fraction with venom PLA_2. These findings bring into question the use of melittin as an activator of endogenous PLA_2 activity, due to the extreme lack of specificity of this action, the high levels of phospholipid hydrolysis due to trace contamination with bee venom PLA_2 and the apparent disruption of the membrane bilayer by the toxin.

Improvements in the Use of Radiolabeled Neurotransmitters in the Studies of PLA_2 Neurotoxin Inhibition of Acetylcholine Release. We are approaching the final stages in improving synaptosome isolation and determination of ACh release. The use of p-bromophenacyl bromide to isolate highly viable synaptosomes and the use of TLC in the analysis of radiolabeled ACh and choline should make the studies on this preparation most informative.

Future Directions.

In the final year of the grant we will examine: (1) the effects of CTX on activation of bee venom PLA_2 hydrolysis of mixed micelles and unilamellar and multilamellar vesicles; (2) the effects of CTX and, to a lesser extent, melittin on activation of endogenous lipolytic enzymes (PLA_2, triglyceride lipase, etc.) using very sensitive assays and preradiolabeled *intact* cells (cell lines); (3) the direct effects of the CTX on tissue lipolytic activity using tissue sonicates and radiolabeled substrates and; (4) toxin internalization using ammonium chloride, methylamine hydrochloride and antibodies to antagonize hemolysis of red blood cells.

The common origin (venom glands), the close association of PLA_2 activity with the mechanism of action and the increased myoplasmic Ca^{2+} and altered Ca^{2+}-regulated K^+ channels demonstrated directly for the B. fasciatus CTX (Figures 22 and 23, respectively) and indirectly for presynaptically-acting snake venom neurotoxins (Dreyer and Penner, 1987) suggest a possible similarity between the primary mechanisms of action of at least some CTXs and

presynaptically-acting neurotoxins. Therefore, we will conduct studies on: (1) effects of antagonists of hemolysis (sphingosine, polymyxin B) on the action of presynaptic neurotoxins on ACh release and; (2) the interaction between ß-Butx and CTX in stimulation and inhibition of ACh release.

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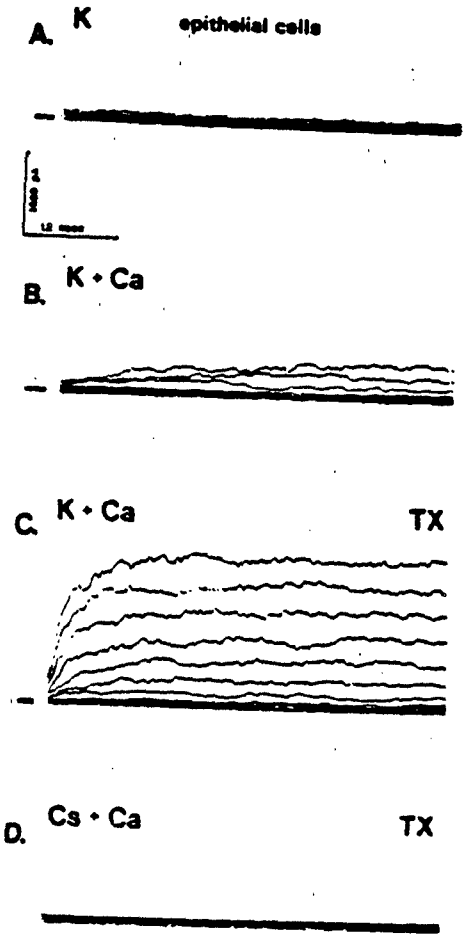


FIGURE 1. Voltage and Ca^{2+}-activated outward currents in transformed airway epithelial cells. (A) Responses with 10^{-8}M intracellular free Ca^{2+}. (B) Responses with 1 µM intracellular free Ca^{2+}. (C) Cell in panel B exposed to 1 µM B. fasciatus toxin for 2 min. (D) Block of current by Cs^{+}, indicating that these are K^{+} currents.

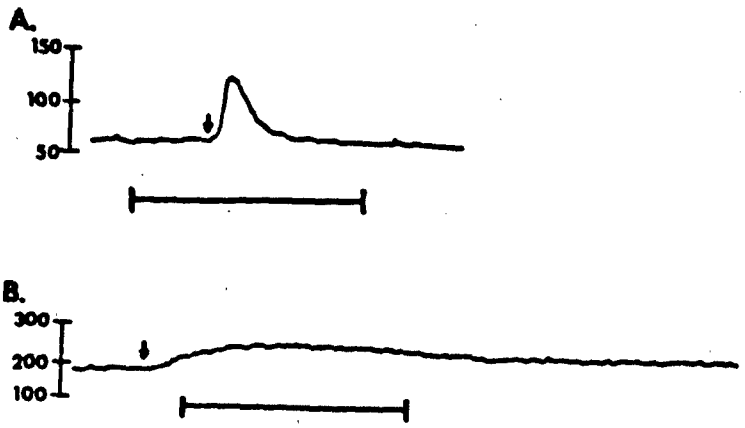


FIGURE 2. Intracellular free Ca^{2+} changes in response to exposure to B. fasciatus toxin. Suspensions of cultured epithelial cells (A) or peripheral blood lymphocytes (B) were loaded with indo-1/AM. The suspension was monitored with toxin (1 µM) was added to the external solution (arrows). The horizontal bars bars indicate a 5 min period. Ca^{2+} levels are in nM and increases are indicated in the upward direction of the Y-axis.

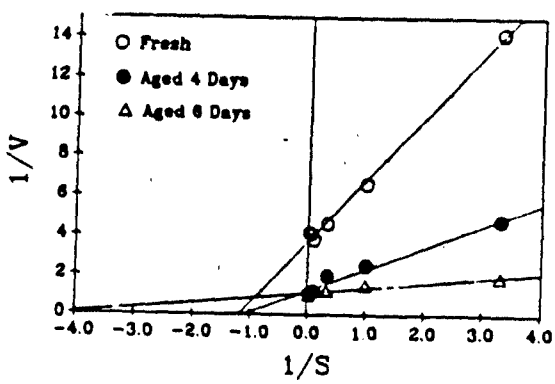


FIGURE 3. Effects of aging on kinetics of hemolysis by N. n. kaouthia CTX.

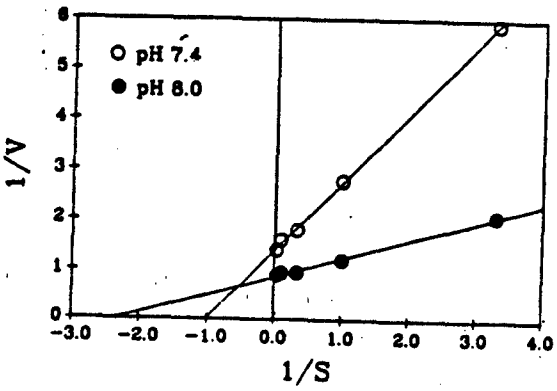
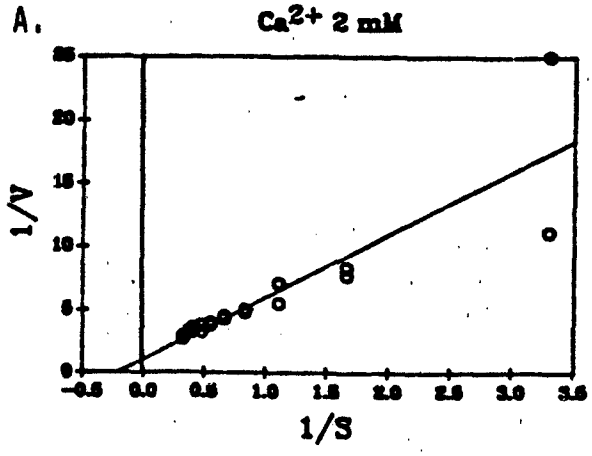


FIGURE 4. Effects of pH on kinetics of hemolysis by N. n. kaouthia CTX.



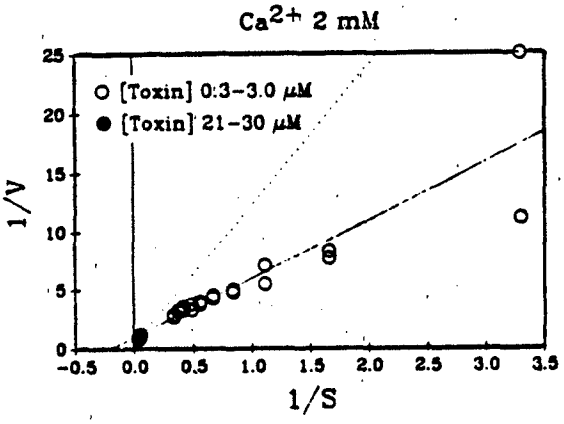


FIGURE 5. Kinetics of hemolysis of low (0.3 - 3.0 μM) and high (21-30 μM) concentrations of N. n. kaouthia CTX.

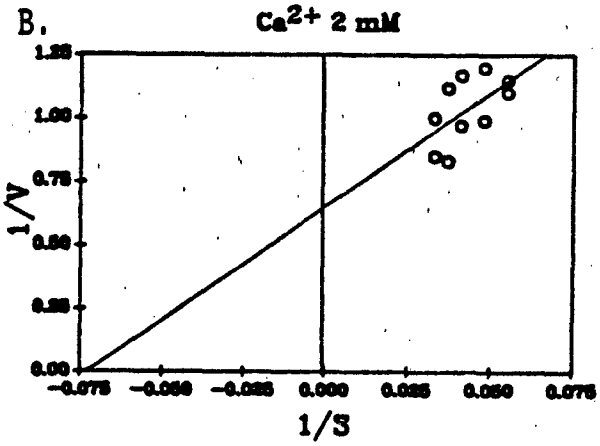


FIGURE 6. Kinetics of hemolysis of and high concentrations of N. n. kaouthia CTX. (A) Hemolysis by low (0.3 - 3.0 μM) concentrations of N. n. kaouthia CTX. (B) hemolysis by high (21-30 μM) concentrations of N. n. kaouthia CTX.

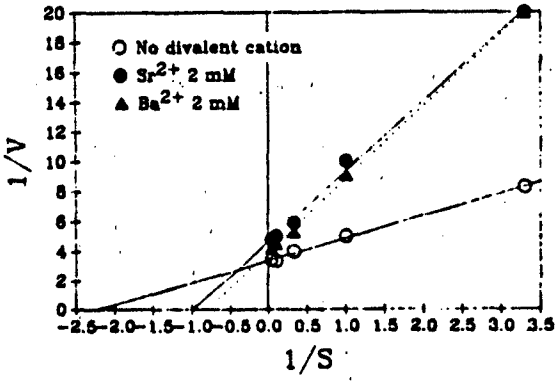


FIGURE 7. Effects of divalent cations on kinetics of hemolysis by N. n. kaouthia CTX. Preparations were either incubated in the absence of divalent cation or in the presence of 2 mM Sr^{2+} or Ba^{2+}

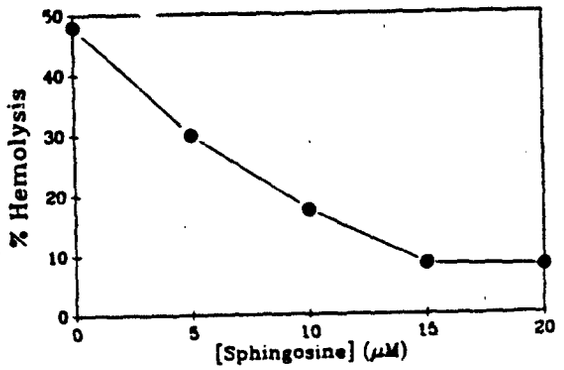


FIGURE 8. The effects of sphingosine on CTX-induced hemolysis. Aged red blood cells were incubated with varying amounts of sphingosine with a fixed concentration (5 μM) of CTX.

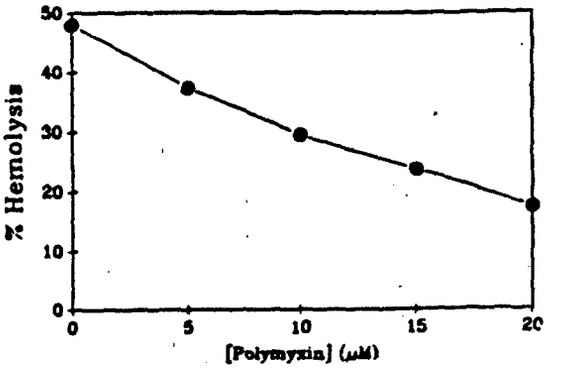


FIGURE 9. The effects of polymyxin B on CTX-induced hemolysis. Aged red blood cells were incubated with varying amounts of polymyxin B with a fixed concentration (5 μM) of CTX.

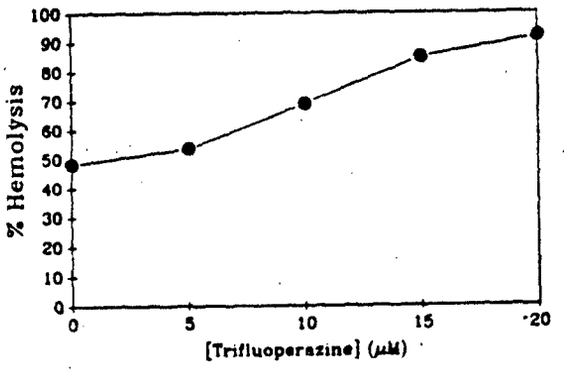


FIGURE 10. The effects of trifluoperazine on CTX-induced hemolysis. Aged red blood cells were incubated with varying amounts of trifluoperazine with a fixed concentration (5 μM) of CTX.

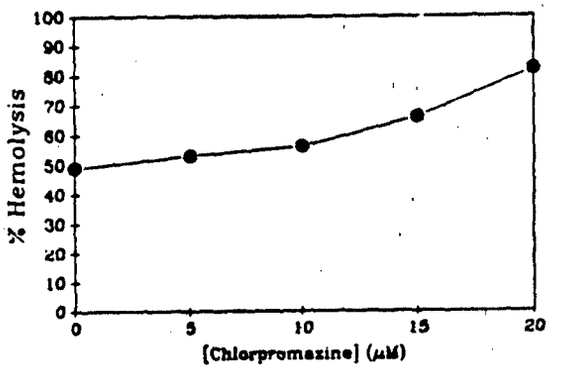


FIGURE 11. The effects of chlorpromazine on CTX-induced hemolysis. Aged red blood cells were incubated with varying amounts of chlorpromazine with a fixed concentration (5 μM) of CTX.

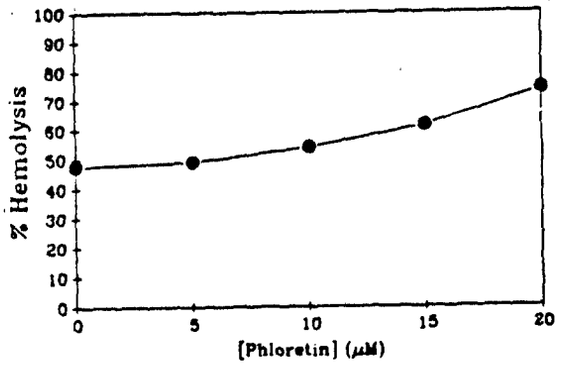


FIGURE 12. The effects of phloretin on CTX-induced hemolysis. Aged red blood cells were incubated with varying amounts of phloretin with a fixed concentration (5 μM) of CTX.

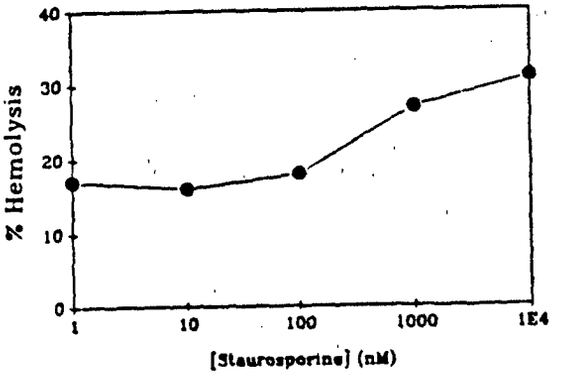


FIGURE 13. The effects of staurosporin on CTX-induced hemolysis. Fresh red blood cells were incubated with varying amounts of staurosporin with a fixed concentration (5 μM) of CTX.

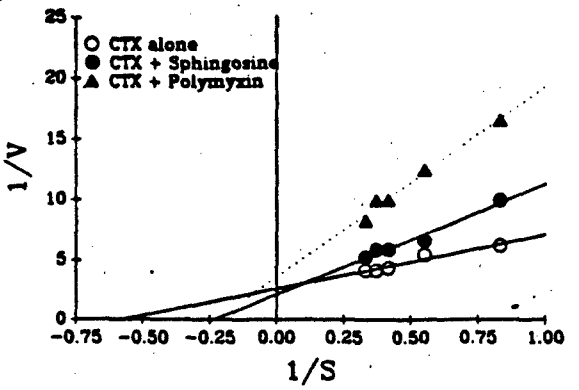


FIGURE 14. The effects of sphingosine and polymyxin B on CTX-induced hemolysis. Aged red blood cells were incubated with varying amounts of sphingosine and polymyxin B with a fixed concentration (5 µM) of CTX and analyzed using a Lineweaver-Burke plot.

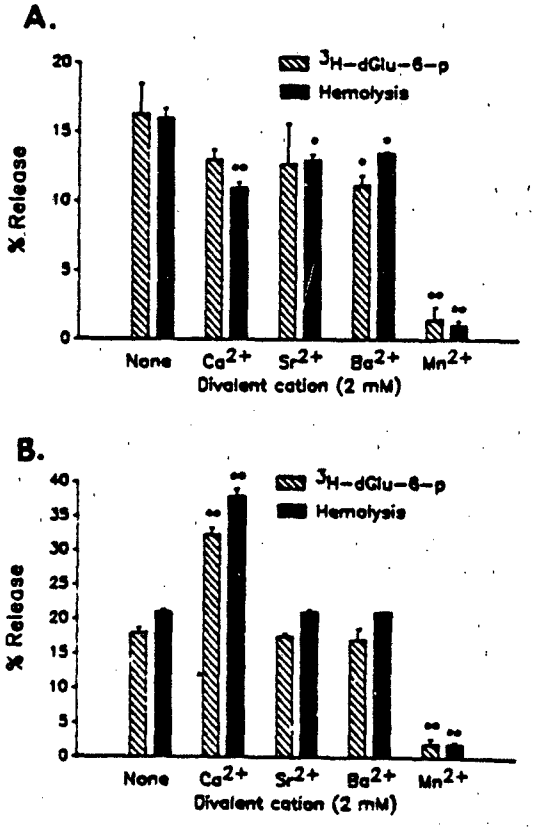


FIGURE 16. The effect of divalent cation type and CTX concentration on hemolysis and ^{3}H-dGlu-6-p release induced by N. n. kaouthia CTX. Erythrocytes were incubated with the CTX for 2 hrs at pH 7.4 and 37°C in a buffer containing a 2 mM concentration of Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, or not containing divalent cation. (A) N. n. kaouthia CTX (3 µM). (B) N. n. kaouthia CTX (10 µM). Values are the mean of triplicate determinations.

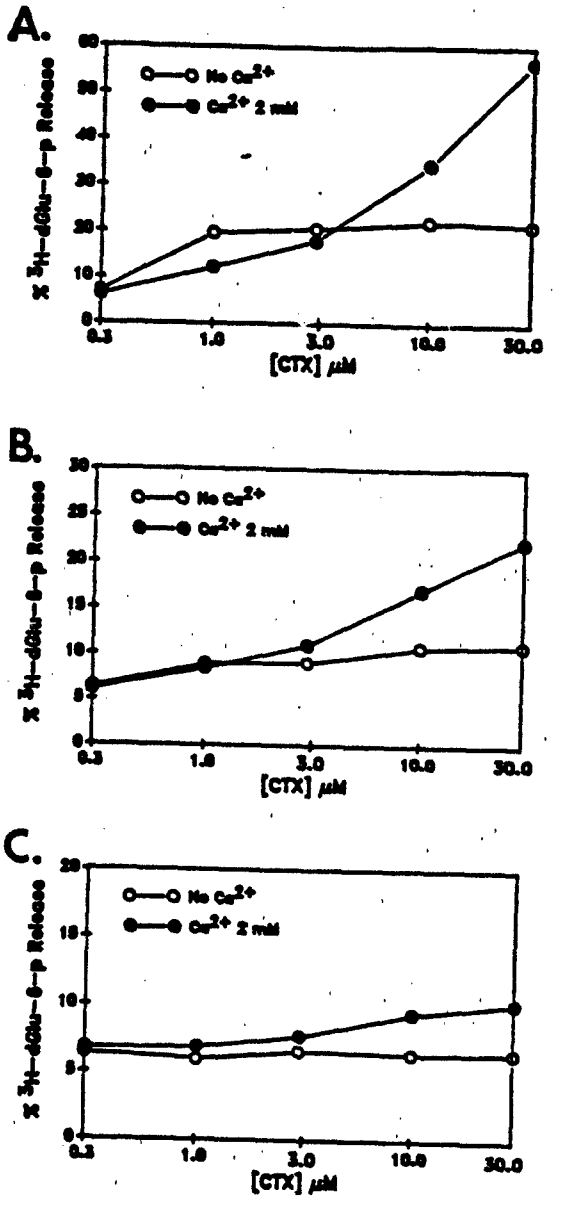


FIGURE 15. Dose response curve of ^{3}H-dGlu-6-p release induced by CTXs from N. n. kaouthia (A) and N. n. atra (B) venoms and a PLA_2 with CTX-like properties from B. fasciatus venom (C) in Ca^{2+} free (unfilled circles) or 2 mM Ca^{2+} (filled circles) media. Red blood cells were preloaded with ^{3}H-dGlu, and incubated with the toxins for 2 hrs at pH 7.4 and 37°C. Values are the mean of triplicate determinations. The SD bars were smaller than the symbols in almost all cases.

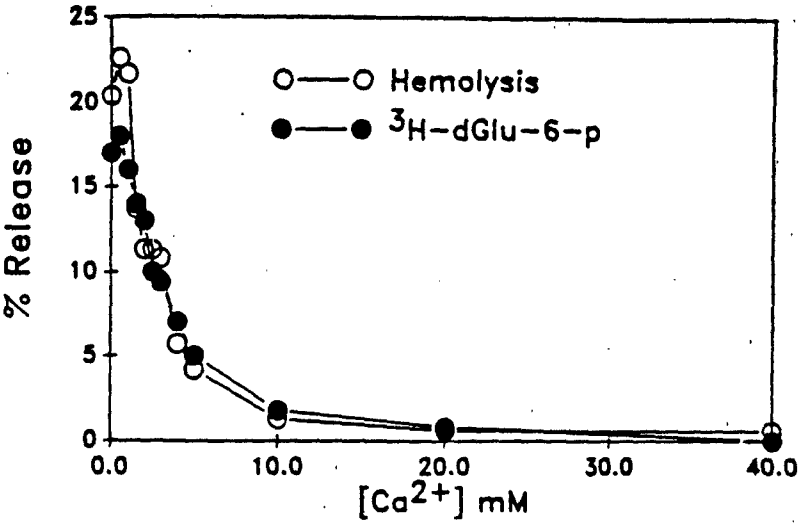


FIGURE 17. Effects of Ca^{2+} concentration in a range of 0-40 mM on hemolysis (unfilled circles) and ^{3}H-dGlu-6-p release (filled circles) by N. n. kaouthia CTX (3 μM). Erythrocytes were incubated with the CTX for 2 hrs at pH 7.4 and 37°C and hemolysis (unfilled circles) or ^{3}H-dGlu-6-p release (filled circles) determined. Values are the mean of triplicate determinations. The SD bars were smaller than the symbols in almost all cases.

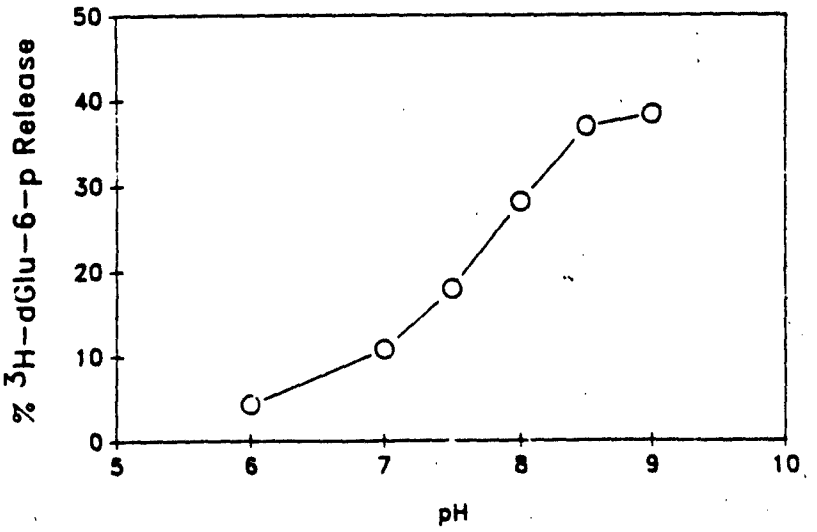


FIGURE 18. The effects of pH on ^{3}H-dGlu-6-p release by N. n. kaouthia CTX (3μM). Erythrocytes were incubated with the CTX for 2 hrs at the indicated pH and 37°C. Values are the mean of triplicate determinations. The SD bars were smaller than the symbols in almost all cases.

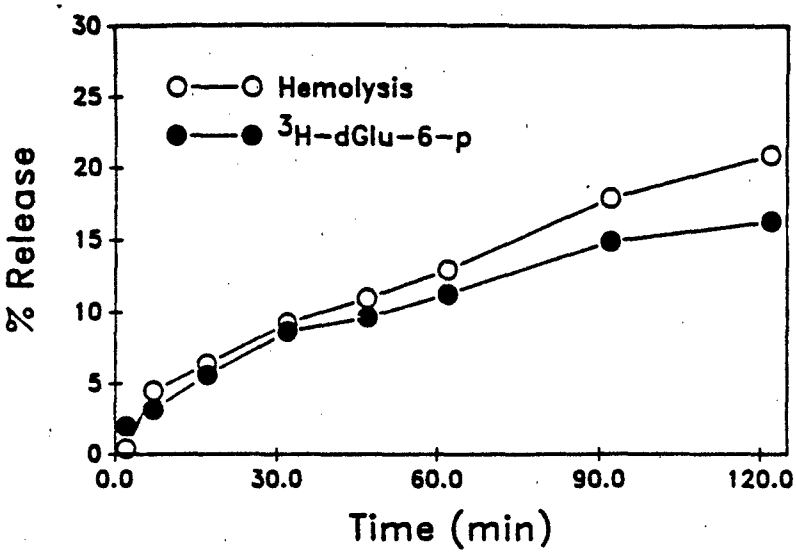


FIGURE 19. Time course of ^{3}H-dGlu-6-p release and hemolysis by N. n. kaouthia CTX (3 µM). Erythrocytes were incubated with the CTX for the indicated time at pH 7.4 and 37°C and hemolysis (unfilled circles) or ^{3}H-dGlu-6-p release (filled circles) determined. Values are mean of triplicate determinations. The SD bars were smaller than the symbols in almost all cases.

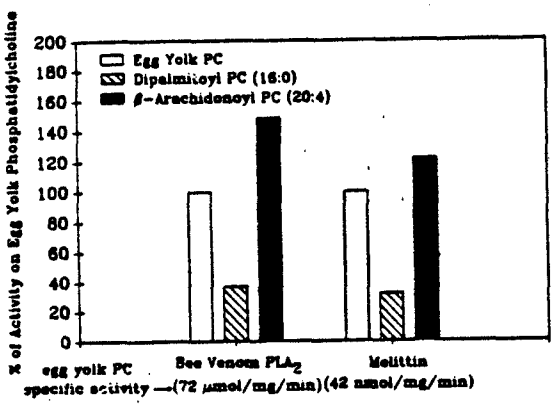


FIGURE 20. Relative PLA2 activities and substrate preferences of bee venom PLA2 and a melittin fraction. Enzyme activities were determined in Tris buffer on PC (1 mM):Triton X-100 (3 mM) mixed micelles in which the phospholipids were: open bars, egg yolk PC; diagonal lines, dipalmitoyl PC, and; filled bars, L-3-PC (2-arachidonoyl, 1-stearoyl). The activities on egg yolk PC were 72 µmol/mg/min and 42 nmol/mg/min, respectively, for bee venom PLA2 (20 ng/ml) and the melittin fraction (200 µg/ml). Values are the mean of two determinations.

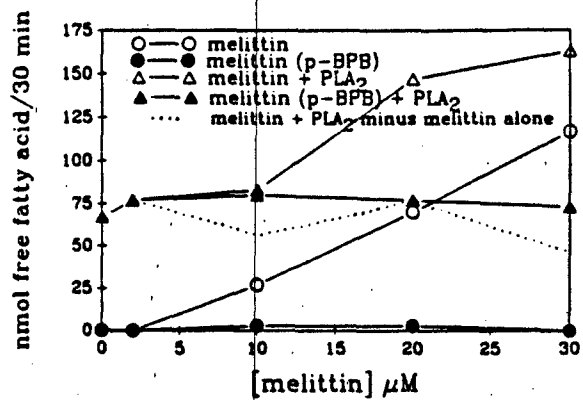


FIGURE 21. Hydrolysis of mixed micelles of egg yolk PC:Triton X-100 (1 mM:3 mM) by bee venom PLA2 and melittin. Fatty acid release for the native melittin fraction (open circles), or the combination of bee venom PLA2 (fixed concentration of 5 nM) and increasing concentrations of the native melittin fraction (open triangles) in Tris buffer. The activity of p-BPB-treated melittin is shown for a single concentration (30 µM; filled circle). The combined activity of bee venom PLA2 and p-BPB-treated melittin is shown for the same concentration of melittin (filled triangle). Values are the mean of three determinations. Error bars (SD) are shown in the case in which the SD values are greater than the size of the symbols.

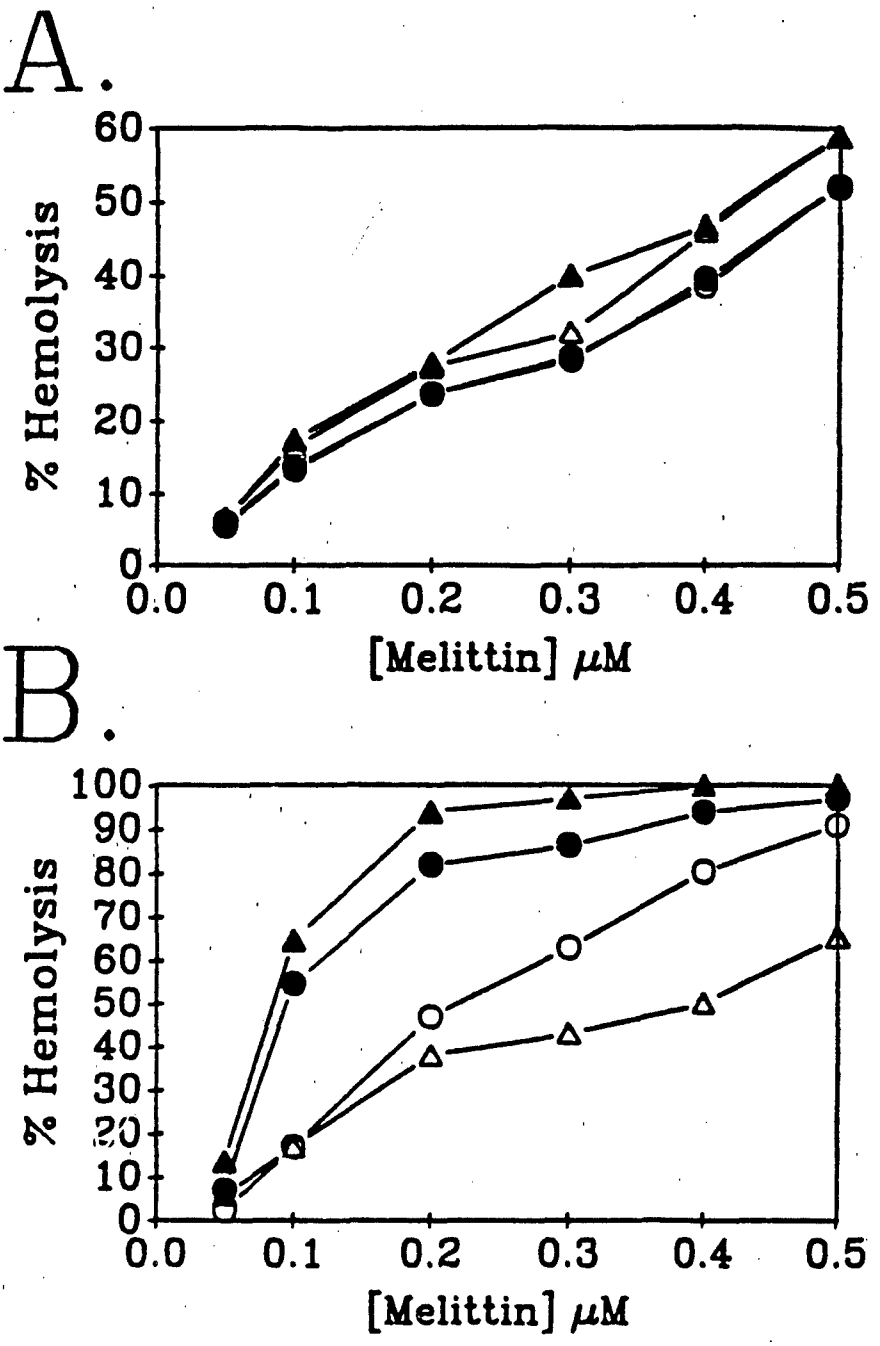
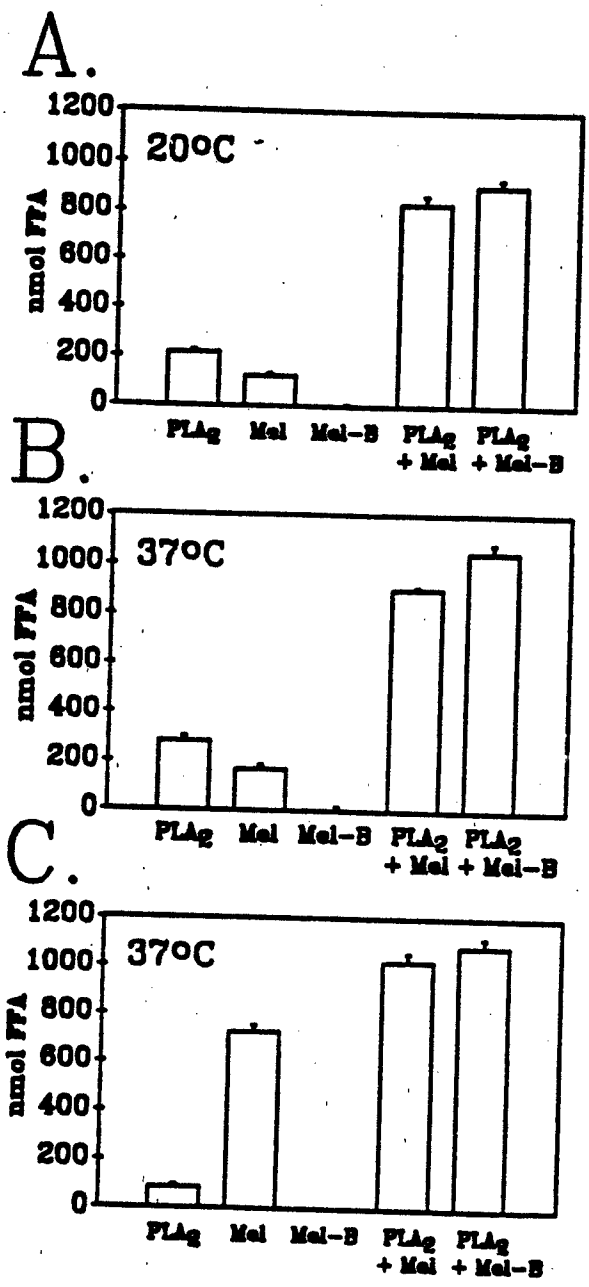


FIGURE 22. Hemolysis induced by native and p-BPB-treated melittin in the absence and presence of bee venom PLA_2. The hemolytic activity was determined (A) in a Ca^{2+}-free medium with EDTA (10 mM), or (B) in a medium containing Ca^{2+} (2 mM). Native melittin (circles) or a p-BPB-treated fraction (triangles) was incubated with red blood cells in the absence (unfilled symbols) or presence (filled symbols) of bee venom PLA_2 (67 nM). Values are the mean for three determination. The SD bars are smaller than the symbols in all cases.

FIGURE 23. Stimulation of bee venom PLA_2 activity on unilamellar and multilamellar egg yolk PC substrates by native and p-BPB-treated melittin. (A,B) Unilamellar substrates and (C) multilamellar substrates were incubated in Tris buffer with bee venom PLA_2, melittin (Mel), p-BPB-treated melittin (Mel-B), or combinations of the native or treated melittin with PLA_2 at the indicated temperatures. The concentrations of melittin and PLA_2 were 5 μM and 30 nM, respectively, for panels A and B. The concentrations of melittin and PLA_2 were 30 μM and 300 nM, respectively, for panel C. Values are the mean±SD for three determinations.



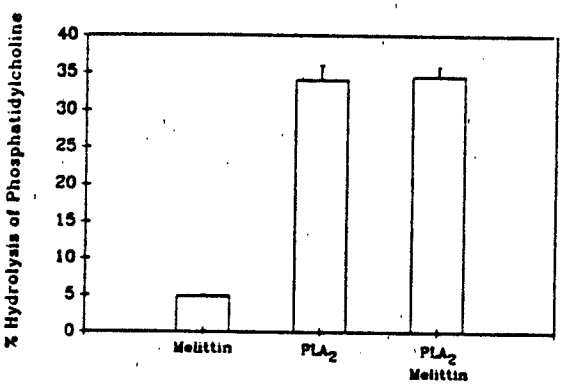


FIGURE 24. Hydrolysis of radiolabeled L-3-PC (1-stearoyl-2-[1-^{14}C]arachidonyl) (10 μM) in mixed micelles of egg yolk PC:Triton X-100 (1 mM:4 mM) by bee venom PLA_2 (1 nM) and melittin (1 μM). Toxins were incubated for 2 hr at 37°C in HEPES buffer. Values are the mean±SD for three determinations.

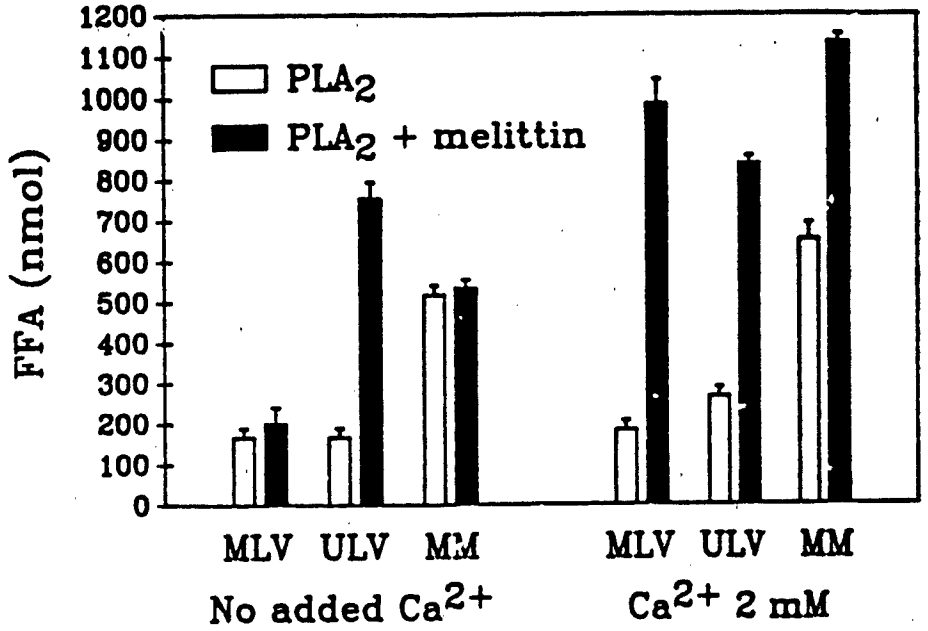


FIGURE 25. Requirement for added Ca^{2+} in the enhancement of enzymatic activity of bee venom PLA_2 by melittin. Multilamellar vesicles (MLV), unilamellar vesicles (ULV) and Triton X-100:phosphatidylcholine mixed micelles (MM) were added to Tris buffer in the absence of added Ca^{2+} (no EGTA added) or in the presence of Ca^{2+} (2 mM) and incubated for 2 hr at 37°C. Fatty acids were titrated by the method of Dole (1956). Bee venom PLA_2 activity in the melittin fraction had been previously inactivated with p-bromophenacyl bromide.

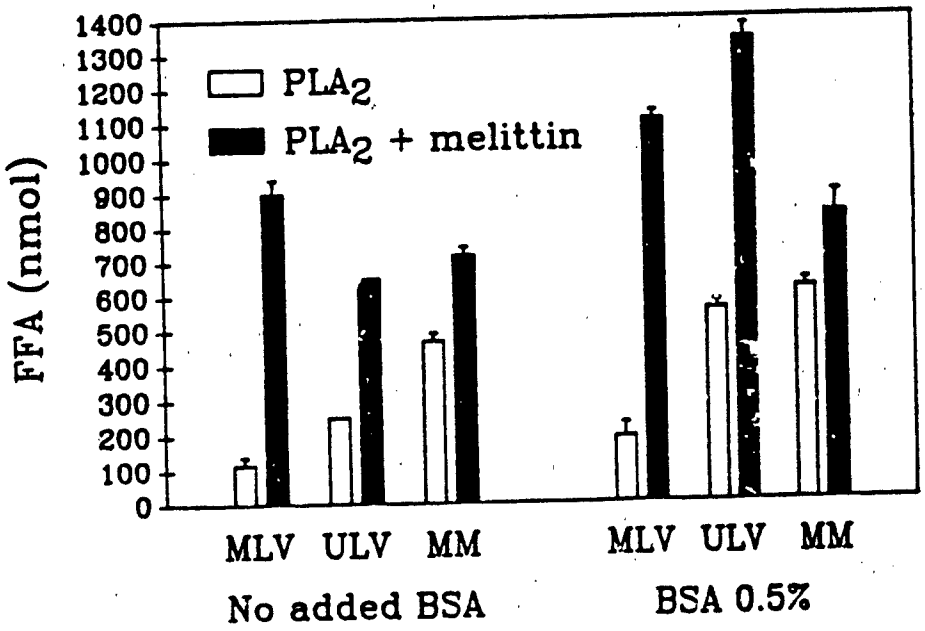


FIGURE 26. Use of BSA to test involvement of lytic products of PLA_2 activity in the interaction between melittin and bee venom PLA_2. MLV, ULV and MM (see Figure 5 for abbreviations) were added to Tris buffer in the absence or in the presence of BSA for 2 hr at 37°C. Fatty acids were titrated by the method of Dole (1956). Bee venom PLA_2 activity in the melittin fraction had been previously inactivated with p-bromophenacyl bromide.

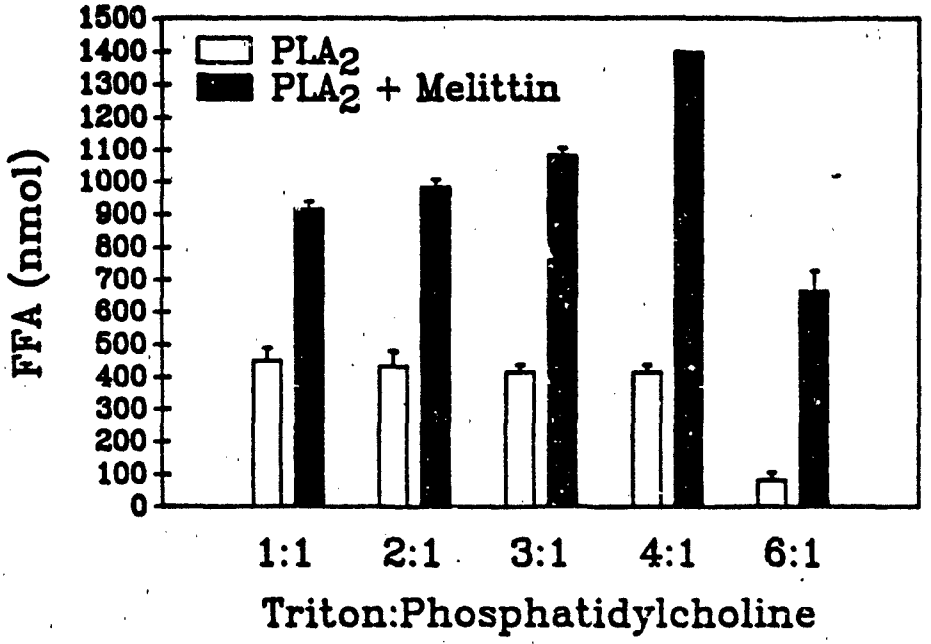


FIGURE 27. Comparison of the effects of Triton X-100 and those of melittin on PLA2 activity in mixed micelles. Mixed micelles of increasing molar ration of Triton:phospholipid were prepared in Tris buffer and incubated with bee venom PLA2 (5 nM) ± p-BPB-treated melittin (10 µM) for 2 hr at 37°C. Fatty acids were titrated by the method of Dole (1956).

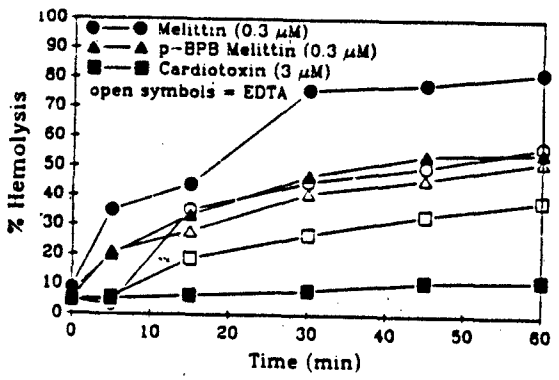


FIGURE 28. Hemolysis induced by melittin, p-BPB-treated melittin and N. n. kaouthia CTX in the absence and presence of EDTA.

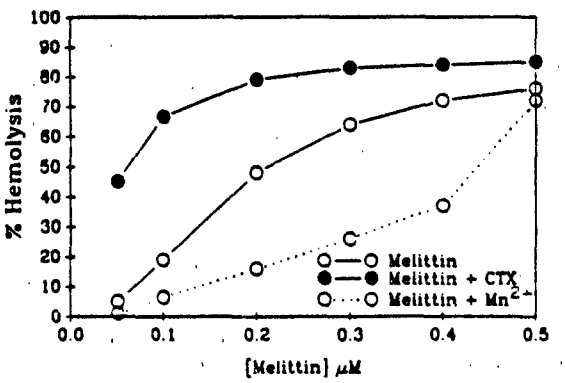


FIGURE 29. Hemolysis induced by melittin in the absence or presence of N. n. kaouthia CTX or Mn^{2+} (2 mM).

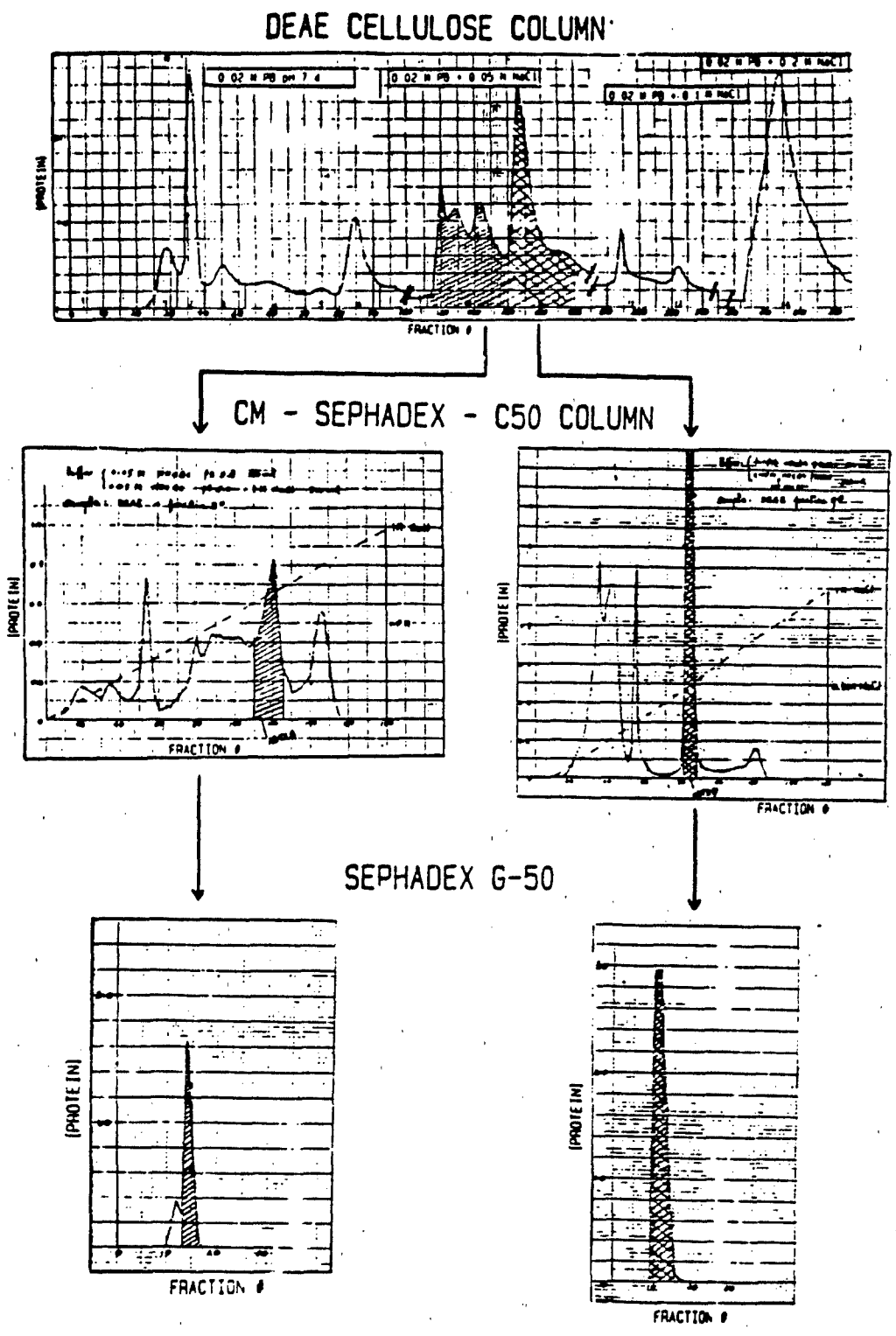


FIGURE 30. Isolation of neurotoxic fractions 8 and 9 from Agkistrodon hallys pallas.

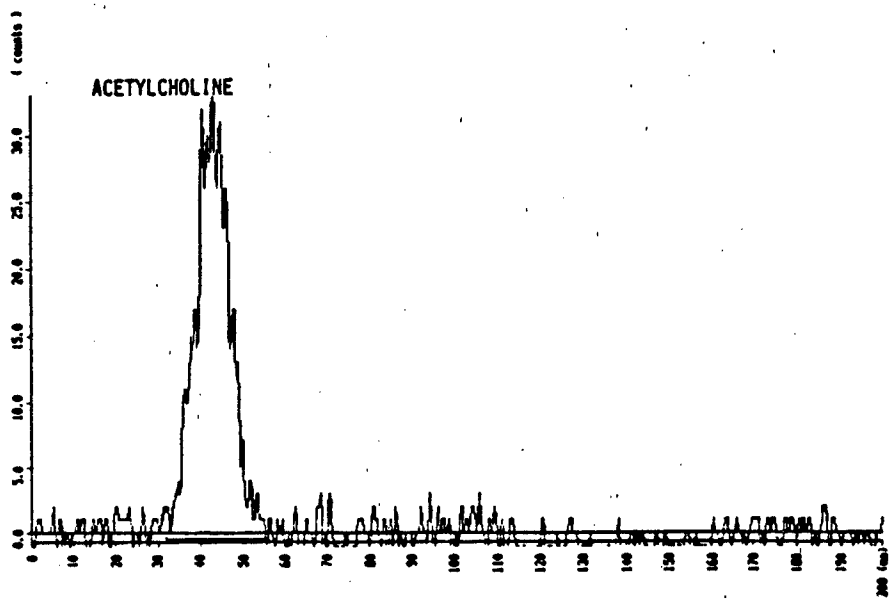


FIGURE 31. Effects of ß-bungarotoxin (0.5 µM) on acetylcholine release from mouse brain synaptosomes. A P_2 fraction was incubated with ß-bungarotoxin for 10 min at 37°C and the samples centrifuged and supernatant lyophilized. The dried sediment was dissolved in methanol, spotted on a TLC plate, developed in one dimension and the radioactivity detected with an imaging scanner. The counts of radioactivity (^{14}C) are indicated in the Y-axis and the distance alone the plate in the X-axis.

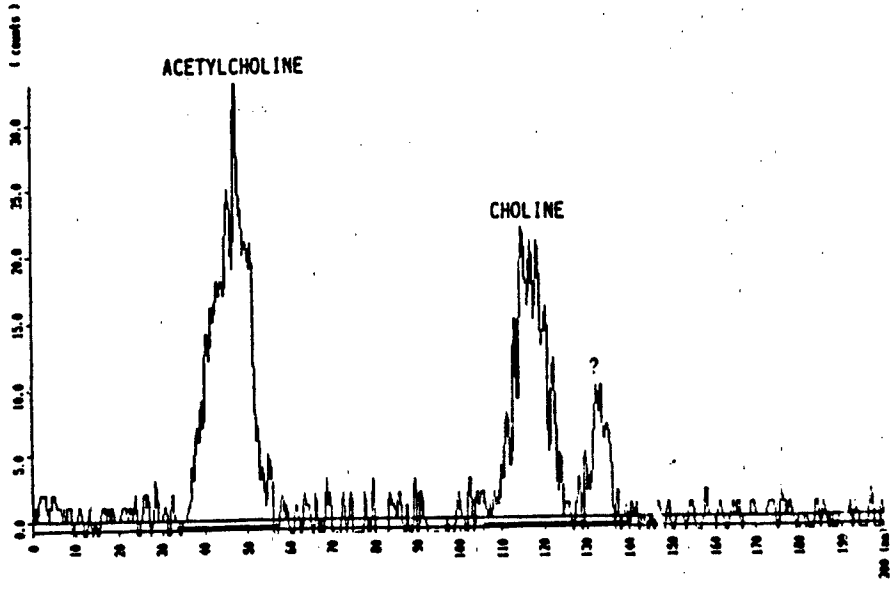


FIGURE 32. Effects of A23187 (10 µM) on acetylcholine release from mouse brain synaptosomes. The conditions are the same as Figure 8. Note that choline is now released and that a minor unidentified component (?) also appears.

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